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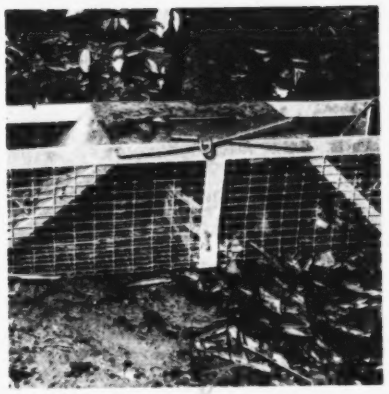
ABSTRACT

Described are techniques used by the National Communicable Disease Center in obtaining blood and tissues from man and other vertebrates for arbovirus isolation and antibody studies. Also included are techniques for capturing and handling vertebrates; banding and marking; restraining and bleeding; storing of specimens to preserve antibody and virus; data recording; and laboratory methods of virus isolation. Consideration is given to planning epidemic surveillance and the selection of a study site. Miscellaneous information is provided on maps, collecting permits, and various types of equipment, both field and laboratory. The use of sentinal and domestic animals to provide blood and tissue samples during known exposures is discussed. Legal, social, political, and technical pitfalls which may severely hamper an arbovirus ecological investigation are identified. An appendix lists the supplies needed, their purpose, sources and approximate costs. Illustrated: short bibliography. (Author/AL)



EDO 45416

# COLLECTION & PROCESSING OF VERTEBRATE SPECIMENS FOR ARBOVIRUS STUDIES

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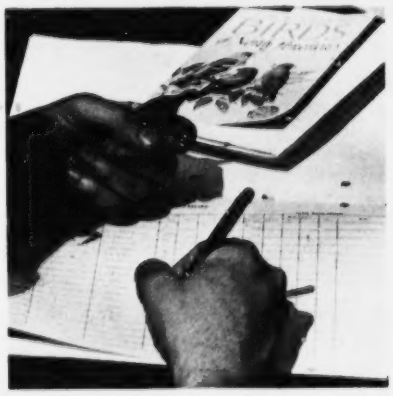

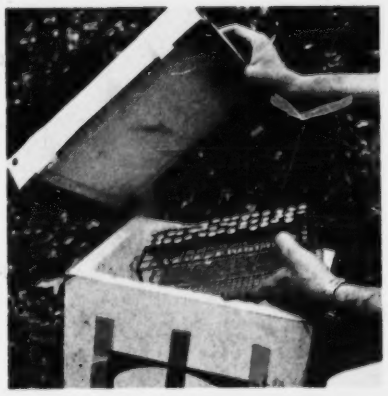

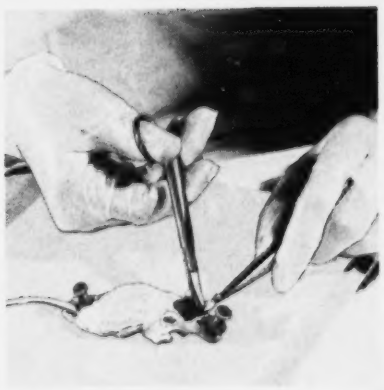

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE • PUBLIC HEALTH SERVICE

EDO 45416

# COLLECTION & PROCESSING OF VERTEBRATE SPECIMENS FOR ARBOVIRUS STUDIES

By

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January 1970

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
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NATIONAL COMMUNICABLE DISEASE CENTER
ATLANTA, GEORGIA 30333

# PREFACE

Wide acceptance by public health workers of "Methods for Collection and Processing of Medically Important Arthropods for Arbovirus Isolation" (1) has encouraged us to prepare a companion manual on vertebrate-arbovirus studies. We hope this second manual will prove as useful as the first. We have drawn largely upon our own experiences and have placed emphasis on methods currently being used by the Arbovirus Ecology Laboratory, NCDC, Atlanta, Georgia, and the Arboviral Disease Section, NCDC, Fort Collins, Colorado. We wish to acknowledge those workers who have shared their knowledge with us and have contributed to the development of the methods described in this manual. Special thanks for their assistance are due Dr. Philip H. Coleman, Dr. Verne F. Newhouse, Dr. Blaine Hollinger, Dr. Roy W. Chamberlain, Mr. J. Gibson Johnston, Jr., Dr. Preston Holden, Dr. Archie D. Hess and Mr. D. Bruce Francy.

## CONTENTS


INTRODUCTION ..... VII
GENERAL CONSIDERATIONS ..... 1
SELECTION OF STUDY SITES ..... 1
COLLECTION AND PRESERVATION OF SPECIMENS ..... 7
HUMAN SPECIMENS ..... 7
FARM ANIMAL SPECIMENS ..... 12
WILD MAMMAL SPECIMENS ..... 13
BAT SPECIMENS ..... 26
BIRD SPECIMENS ..... 28
REPTILE AND AMPHIBIAN SPECIMENS ..... 46
LABORATORY PROCEDURES ..... 49
LITERATURE CITED ..... 60
APPENDIX ..... 62

# INTRODUCTION

Arbovirus investigations require specialized techniques because of the relatively complicated virus-vector-host relationships. Although these relationships may vary with the specific virus or geographical area involved, the basic investigational techniques are similar. The detection of endemic foci, surveillance for early epidemic activity, or other arbovirus field studies generally include the testing of vertebrate hosts for the presence of virus or antibody.

The main purpose of this manual is to explain and illustrate techniques used by NCDC in obtaining blood and tissues from man and other vertebrates for arbovirus isolation and antibody studies. Included are the techniques for capturing and handling of vertebrates; banding and marking; restraining and bleeding; storing of specimens to preserve virus and antibody; recording of data; and laboratory methods used for virus isolation. Consideration is given to planning epidemic, surveillance, and special studies and the selection of a study site. Miscellaneous information is also provided on maps, collecting permits, and various types of equipment.* The use of sentinel and domestic animals to provide blood and tissue samples during known exposure periods is also discussed. Finally, the reader is cautioned concerning legal, social, political, and technical pitfalls which may severely hamper an arbovirus ecological investigation.

*The use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U. S. Department of Health, Education, and Welfare.

# GENERAL CONSIDERATIONS

Comprehensive arbovirus field studies require the *coordinated* effort of many scientists: physicians, epidemiologists, veterinarians, ecologists, mammalogists, ornithologists, entomologists, statisticians, and virologists. The virologist should have well equipped laboratory facilities. Of course, limited studies can be investigated by one or more of these specialists; but regardless of the scope, the study must be well planned with realistic objectives compatible with field and laboratory capabilities.

Usually the public health officer of the area assumes or delegates the responsibility for an arbovirus research program and obtains the advice of his staff and experienced arbovirus workers in formulating a plan of action designed to reach specific objectives. These objectives may be formulated by stating them in terms of questions to be answered either qualitatively or quantitatively. After reviewing the work in progress and analyzing the data collected, the research team may need to ask additional questions and expand the original objectives.

Some qualitative questions might include: "Has there been any arbovirus activity in the area," and if so, "Which viruses were active?" "Which are active at the present time?" "Which vertebrates are involved as hosts?" "Which vector is responsible for the virus transmission?". After these questions are answered, quantitative aspects may be investigated. "How much virus activity is there or has there been in the human population?" "What are the infection rates in mosquitoes, birds, or mammals as measured by virus isolation or antibody studies?"

Later, even more complicated objectives may be pursued. "How is virus amplification, transmission, distribution, and maintenance achieved?" "If the virus is not endemic in the area, what is the method of reintroduction?" "Can surveillance for virus or antibody in immature birds be used to detect early virus activity in the community?" "After virus activity has been detected, when and what kind of control procedures should be initiated?"

Obviously, reaching any of these objectives requires team work between laboratory and field personnel. In addition, the success of any field program depends to a great extent upon good public relations and presenting sufficient information to government officials, landowners, and the general public to gain their acceptance.

Finally, due consideration must be given to developing an effective safety program for all workers to protect them from infection. Also, care should be taken to avoid the contamination of specimens which may cast doubt on the validity of the results.

## SELECTION OF STUDY SITES

The first step in the selection of a study site is locating an area in which arbovirus activity is manifested. Direct evidence of activity is provided by human or animal cases during an epidemic or epizootic or by the isolation of an arbovirus from a wild animal host or arthropod. Indirect evidence of virus activity may come from serum-antibody surveys of man or other vertebrate hosts, indicating that infection has occurred in the general area. Once a favorable area has been located, the second step is to select one or more small workable areas, often no more than a few acres in size within the large area under consideration. Many sites may have to be examined before suitable ones are found.

### Use of Epidemiological Data

A favorable geographical area for study is delineated during an epidemic by the occurrence of human cases, which serve as an index of recent virus activity. However, since the incubation period of the virus in humans

may be as long as three weeks, the occurrence of a case does not necessarily indicate "current" virus activity. Also, the patient's exposure to the arthropod vector may have occurred at any one of a number of locations other than his residence. These considerations and the probability that vector control measures will be aimed at the patient's neighborhood may preclude productive studies in these immediate areas. Nevertheless, the locations of human cases can be plotted on a map, and the most recent case, or cluster of cases used as a starting point in the search for suitable sites. Then a preliminary field survey is made to determine the most favorable sites for starting work.

During an epidemic, it may be necessary to limit bird or mammal collections to only one or two study sites. However, after the epidemic, additional surveys encompassing a wider area can be made to determine the extent of the area involved.

Another index of recent arbovirus activity may be an epizootic in domestic animals or birds. Early recognition of epizootics is important since human cases sometimes follow within one or two weeks. For eastern or western encephalitis, bird studies at or near a farm where cases occurred can be productive if the virus is still in the amplification phase. Grains and other foods usually available around farms attract many birds, and quite often a high population of vectors may be present as well. Vector control is less likely to interfere with studies at farm sites, increasing the probability of detecting further virus activity.

Reports of human or horse cases of encephalitis from an area over a period of years indicate endemicity of the virus there, either through perennial maintenance or annual reintroduction. Such a study site is more likely to yield information on basic infection cycles and factors influencing spread than an area without comparable epidemiological information.

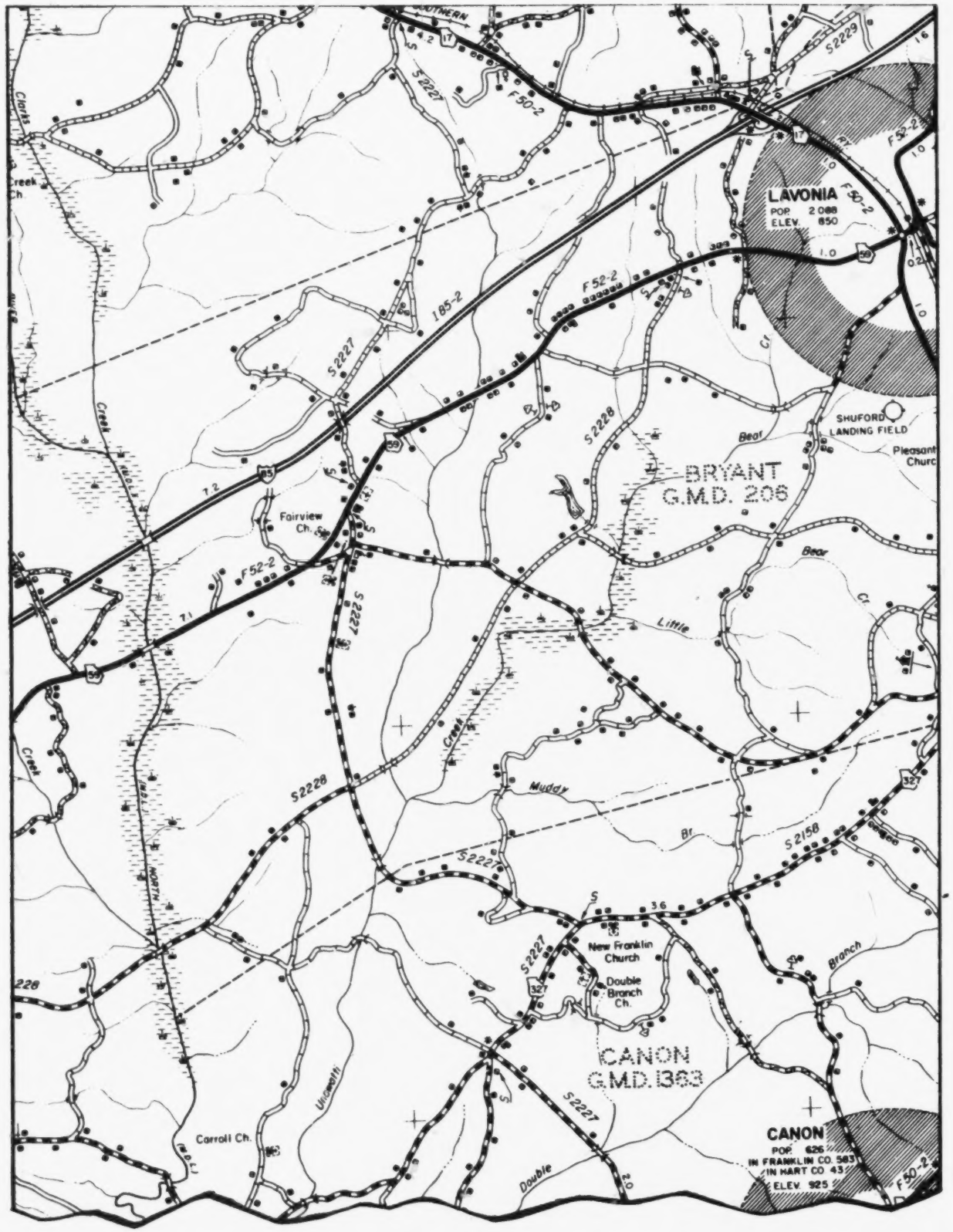
Information obtained from antibody surveys must be carefully evaluated when it is to be used as a basis for study site selection. Detection of antibody indicates *what kind* of arbovirus activity took place, but does not necessarily indicate *when* or *where* the activity occurred or whether the virus is still active in the area. More specific information can be derived if samples are taken from immature animals rather than from adults.

If a study site fails to yield satisfactory data, it should be abandoned in favor of a more productive location. The following example illustrates this principle. A serological survey of the Seminole Indians of Big Cypress Reservation, Florida, showed that 58% had Venezuelan encephalitis (VE) antibodies (2). Both mosquito and wild vertebrate studies were intensively conducted at the Reservation for a year in attempts to isolate VE virus and confirm the serological findings, but without success. Then the studies were shifted 30-50 miles to the south. Over 50 different sites were sampled and VE virus was recovered from vectors or hosts in four of them. These locations were selected for continuing studies.

**Use of Maps**

State highway department maps and oil company road maps are useful, but more detailed county or city maps may be obtained from the county courthouse or the city hall (**Figure 1**). U. S. Geological Survey topographic map indexes, published for each state, Puerto Rico, the Virgin Islands, Guam and American Samoa are available free upon request to the U. S. Geological Survey, Washington, D.C. 20242, or the Federal Center, Denver, Colorado 80225. Appropriate quadrangles (**Figure 2**) may be ordered from these offices at nominal cost. The maps also may be purchased at sporting goods stores in some areas. Usually the most desirable scale is the one showing the greatest detail of the area.

Aerial photographs are useful as an aid in selecting a field site (**Figure 3**) since they show details of drainage systems, types of forested areas and other land uses, and features often quite difficult to see from the ground. Aerial photographs may be obtained or viewed at local or state offices or road departments.




# LEGEND

## BOUNDARIES

- STATE
- COUNTY
- MILITIA DISTRICT (G.M.D.)
- CORPORATE LINE
- NATIONAL OR STATE PARK
- NATIONAL OR STATE FOREST
- NATIONAL OR STATE MONUMENT
- AIRPORTS, MILITARY AND NAVAL RES.

## RAILROADS

- SINGLE OPERATING COMPANY
- MORE THAN ONE OPERATING COMPANY
- NARROW GAGE RAILROAD
- STANDARD GAGE, PRIVATELY OWNED
- NARROW GAGE, PRIVATELY OWNED
- RAILROAD ON STREET OR ROAD
- RAILROAD GRADE CROSSING
- UNDERPASS – HIGHWAY BELOW
- OVERPASS – HIGHWAY ABOVE
- RAILROAD STATION
- RAILROAD BRIDGE

## NAVIGATION

- NAVIGABLE STREAM
- HEAD OF NAVIGATION
- OPERATED SHIP OR BARGE LINES
- WHARF, DOCK, PIER OR LANDING
- LIGHTHOUSE
- LIGHT, NAUTICAL
- FERRY (T.F TOLL – FF FREE)
- CANAL
- DAM WITH LOCK

## DRAINAGE

- INTERMITTENT STREAM
- NARROW STREAM OR CREEK
- WIDE STREAM OR RIVER
- DRAINAGE DITCH
- RESERVOIR, WITH DAM
- RESERVOIR, LAKE OR POND
- SWAMP OR MARSH

## STRUCTURES - NARROW STREAM

- MINOR STRUCTURES 10' TO 20' SPAN
- HIGHWAY BRIDGE – OVER 20' SPAN
- SERIES SHORT SPAN BRIDGES

## STRUCTURES - WIDE STREAM (T-TOLL)

- HIGHWAY BRIDGE, GENERAL
- DRAWBRIDGE
- SUSPENSION BRIDGE
- ARCH
- TRUSS (W-WOOD, S-STEEL)
- COVERED BRIDGE

## MISCELLANEOUS STRUCTURES

- DAM
- DAM WITH ROAD
- LEVEE OR DYKE
- LEVEE OR DYKE WITH ROAD
- PASS
- FORD
- CATTLE GUARD
- TRAFFIC CIRCLE INTERSECTION
- CLOVER LEAF INTERSECTION

## ROAD SYSTEM DESIGNATION

- UNITED STATES HIGHWAY
- STATE HIGHWAY
- [illegible]

## CITY AND VILLAGE CENTERS

- STATE CAPITAL
- COUNTY SEAT
- INCORPORATED CITY (POP 1960 CENSUS) (ELEVATION, FEET ABOVE SEA LEVEL)
- DELIMITED AREA
- UNINCORPORATED TOWN OR CITY CENTER

## CULTURE SYMBOLS

| | IN USE | NOT IN USE |
|---|---|---|
| FARM UNIT | | |
| DAIRY | | |
| DWELLING | | |
| GROUP OF DWELLINGS | | |
| NURSERY | | |
| WAREHOUSE OR LARGE BARN | | |
| SCHOOL OR COLLEGE | | |
| CEMETERY | | |
| CHURCH & CEM ADJACENT, CHURCH | | |
| HOSPITAL | | |
| COMMUNITY AUDITORIUM | | |
| POST OFFICE | | |
| STATE POLICE DISTRICT HEADQUARTERS | | |
| CORRECTIONAL INSTITUTION | | |
| COUNTY PRISON FARM | | |
| SEASONAL DWELLINGS | | |
| FRUIT OR NUT ORCHARD | | |

## BUSINESS AND INDUSTRIAL SYMBOLS

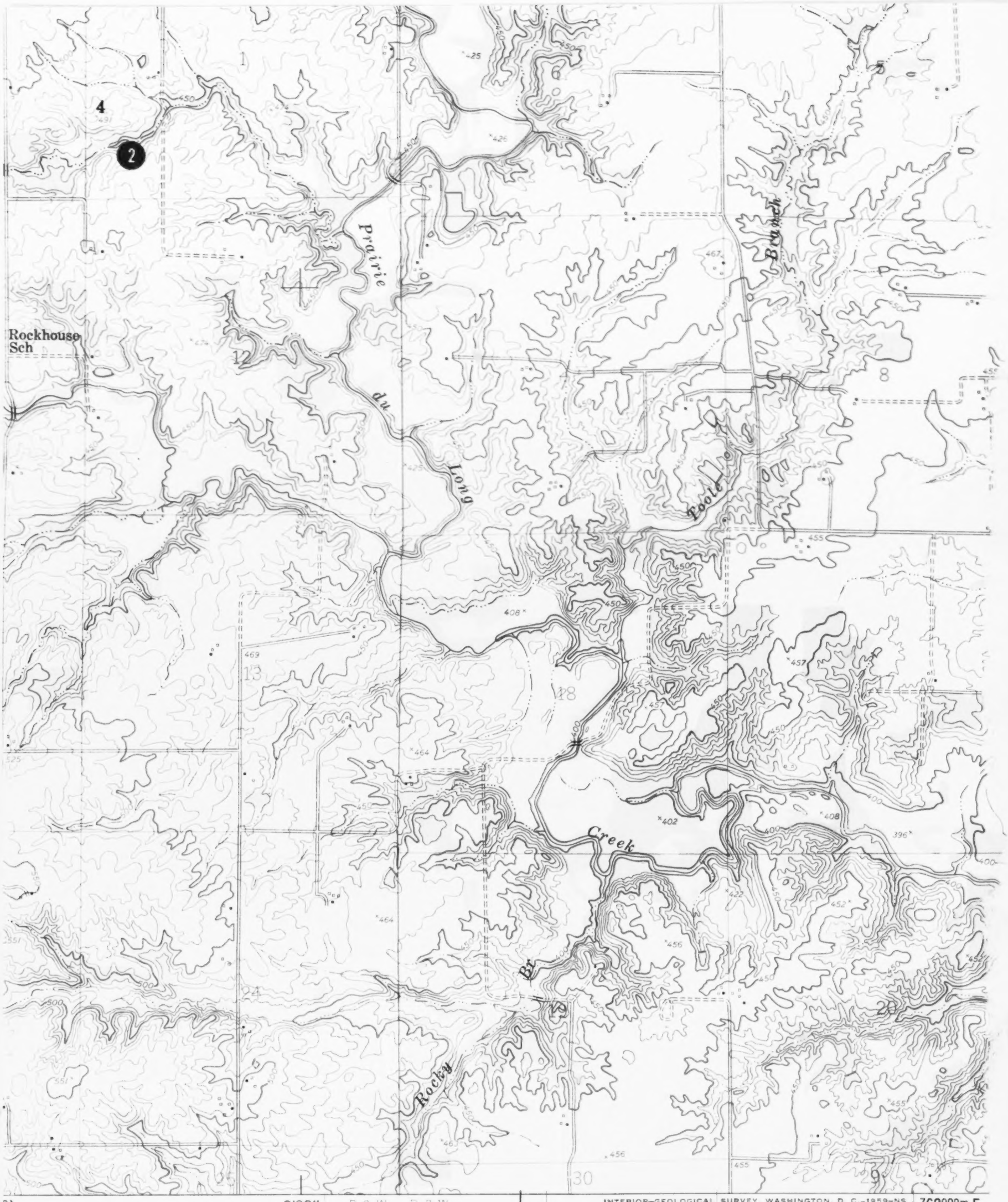
- STORE OR SMALL BUSINESS
- GROUP OF STORES
- BUSINESS AND HOME COMBINED
- BUSINESS AND POST OFFICE
- BUSINESS AND FILLING STATION
- FILLING STATION
- TOURIST CAMP
- HOTEL
- FACTORY OR INDUSTRIAL PLANT
- POWER PLANT
- SEASONAL INDUSTRY
- SAW MILL – STATIONARY
- FRUIT PACKING PLANT
- TURPENTINE STILL
- RADIO STATION (WSB)
- TANKS
- SEWAGE DISPOSAL PLANT

## RECREATIONAL FEATURES

- DRIVE-IN THEATRE
- GOLF COURSE OR CLUB
- SMALL MONUMENT
- WAYSIDE PARK OR AMUSEMENT PARK (WP)
- MUNICIPAL PARK
- FAIR GROUND OR RACE COURSE
- FORT
- LODGE OR CAMP HOUSE
- CAMP GROUND
- PICNIC GROUND
- PLAY GROUND OR BALL PARK
- BATHING BEACH
- SCENIC SITE
- BIRD SANCTUARY
- GAME PRESERVE
- FISH HATCHERY
- FISHING AREA

## MISCELLANEOUS SYMBOLS

- PROMINENT ELEVATION (FEET ABOVE SEA LEVEL) (1257)
- COAST GUARD STATION
- LIFE SAVING STATION
- FOREST RANGER STATION
- OBSERVATION OR LOOKOUT TOWER




1 MILE

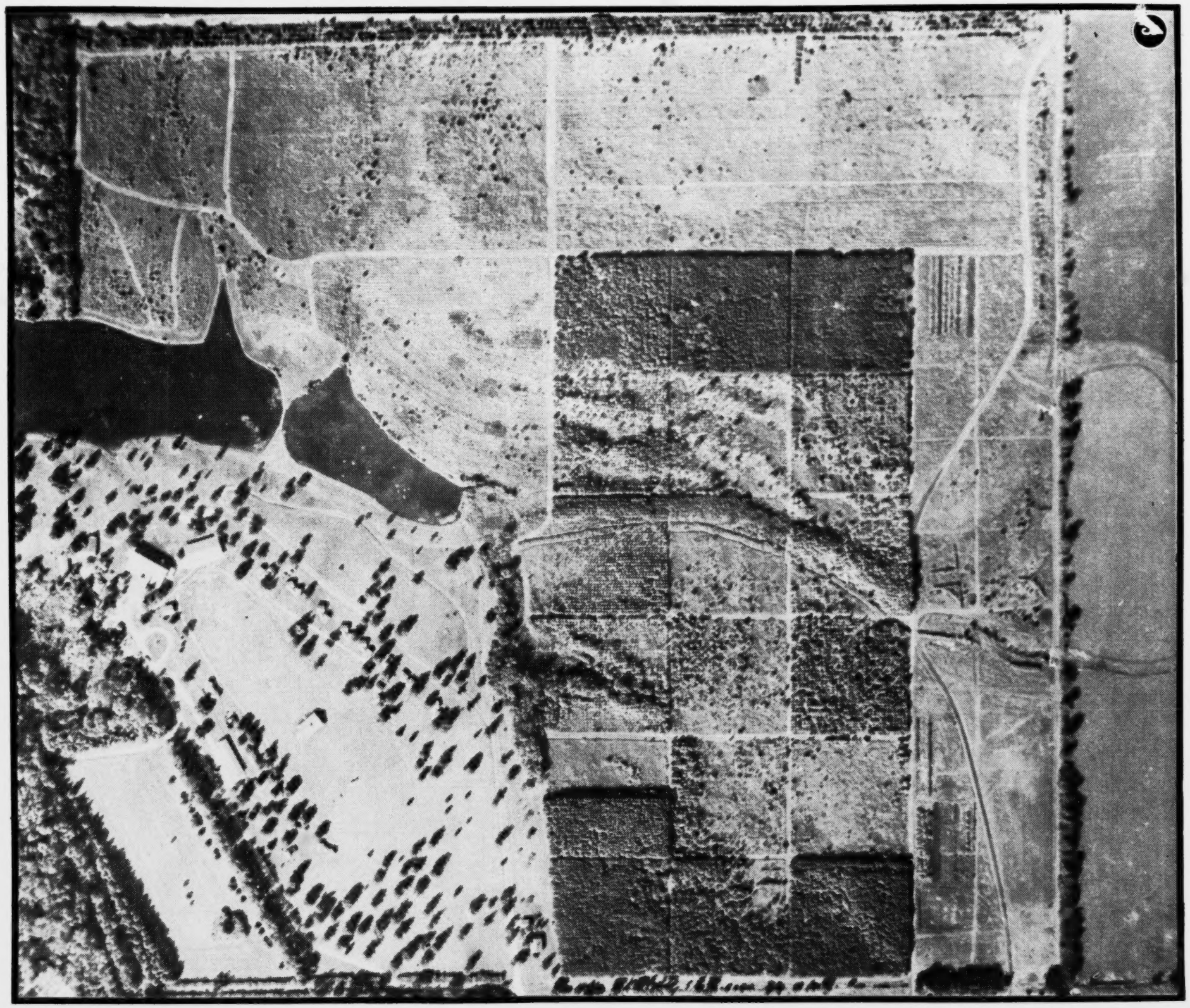
00 5000 6000 7000 FEET

1 KILOMETER

ROAD CLASSIFICATION

Heavy-duty — Light-duty

Medium-duty — Unimproved dirt



**Survey of Potential Study Sites**

The success or failure of a field program may depend upon the sites finally chosen for study. It is important, therefore, to make this decision carefully and, if possible, with personal knowledge of the area.

When a study is started, many kinds of vertebrates are generally collected; therefore, a variety of habitats should be present in the area to favor the capture of as many species as possible. The sites selected should reflect the generalized habitats of the region. However, altered or unique habitats should not be ignored since they may increase the potential for buildup of host or vector population.

The largest populations of bird and mammal species usually occur at the edges between two types of habitats, which offer protective cover and greater food selection. Trapping along these edges can yield higher capture rates and a wider range of species than either habitat alone.

After determining presence of the desired animal species, it is necessary also to determine that opportunity exists for appropriate vector-host association.

No site should be used without permission from the proper authority. One advantage of using privately owned land is that some privacy is assured, precluding the disturbance of collecting equipment. When using public lands, it is advisable to select seldom frequented sites for the same reason. However, easy access is important since field studies may require transport of bulky trapping equipment.

Consideration should also be given to seasonal variations in bird, mammal and vector populations. Anticipation of seasonal changes in the area is a help in planning workable time schedules.

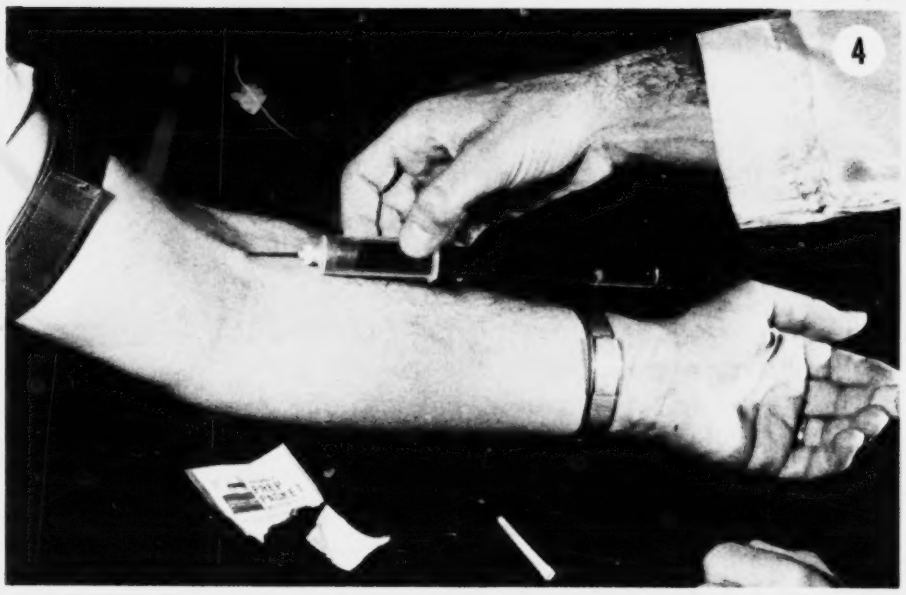
A considerable amount of time, money and energy may be expended during a field study. A preliminary survey to determine the feasibility of the project helps to protect this investment. The choice of the study area and sites deserves much forethought and a preliminary survey as basis for final judgment.

**Equipment Required**

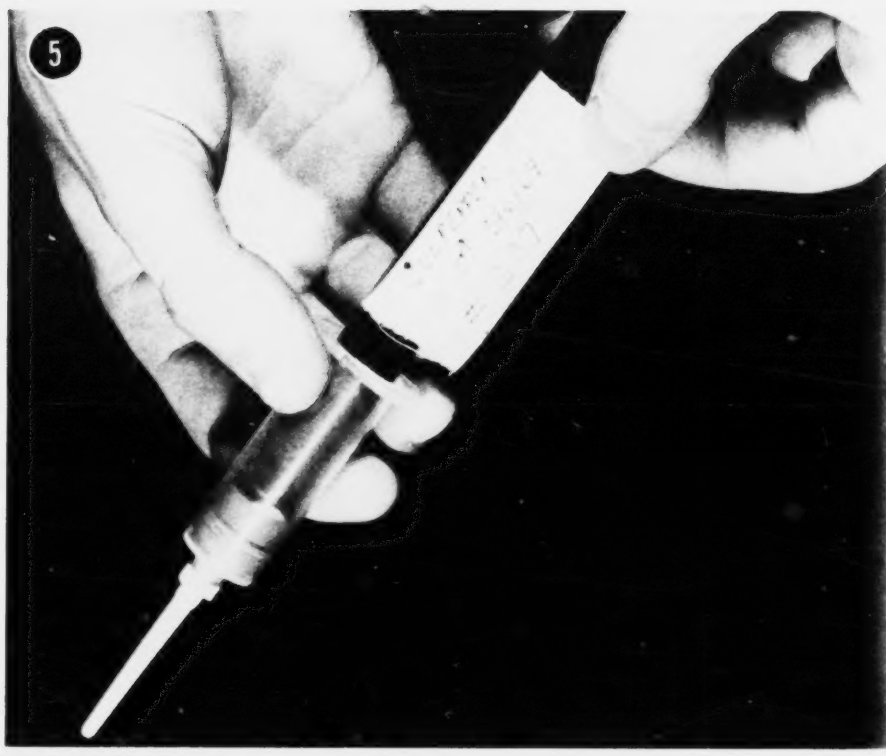
Supplies needed to conduct an antibody survey are listed on page 62 in the Appendix; the amount of each item required depends on the number of blood samples desired.

**Taking the Blood Specimens**

Using aseptic techniques (**Figure 4**), 20 ml of blood are collected by venipuncture from every person over the age of two; 10 ml are obtained from those less than two years old. In bleeding very small children, a disposable syringe and needle may yield greater quantities of blood than a 20 cc Vacutainer, which may cause collapse of the vein. No anticoagulants or preservatives are used, but precautions should be taken against contamination. Firmness, self-assurance and kindness are essential for cooperation from the younger age group.




Each specimen should be given an adhesive tape label (**Figure 5**) as soon as taken, specifying the person's name, age, race, sex, the date of collection and a field number corresponding to that on the interview sheet (**Figure 6**). Use a soft-lead pencil or ballpoint pen with waterproof ink. Laying the tube on its side for an hour at ambient temperature increases the serum yield. Then the tube should be kept cool and out of direct sunlight during the working day.




**Data Recording**

While the physician or nurse collects the specimen, the assistant records the pertinent interview data (**Figure 6**). Since a single data sheet covers an entire family unit, duplication of last names and other common information is unnecessary. This form was designed for data processing and statistical analysis. Information regarding places and duration of residence, military history, yellow fever vaccination or foreign travel (for longer than three months) is necessary when attempting to establish the source of specific antibodies. Further residence histories are entered on the reverse side of the sheet (**Figure 7**). Any localities where an individual has resided six months or longer should be listed with year of residence and the total time should agree with the length of residence given on the front side. The field number represents any consecutive coding system chosen to identify a specimen until it receives a permanent laboratory number. Errors are avoided by double-checking the specimen number with the interview sheet before proceeding to another donor.

Information on the occupation and education of the household head is requested for sociological classification. Questions relate to

# COLLECTION AND PRESERVATION OF SPECIMENS

## HUMAN SPECIMENS

### General Considerations

A major goal of arbovirus field research is to determine the extent of human infection. In epidemics, current human infection by a particular virus can be determined by the isolation of the virus from a clinically ill patient, or by the demonstration in paired sera (acute vs convalescent) of a four-fold or greater rise or fall in specific complement fixation (CF) or hemagglutination-inhibition (HI) antibodies. Surveys involving single (non-paired) sera are primarily conducted to determine the proportion of the people in the community immune to the suspect virus infection.

In non-epidemic periods, on the other hand, the purpose of serological surveys may be to determine which of various viruses have been active in the area. For this purpose a large number of single serum samples are tested against a battery of several selected antigens.

### Communications

Once the general area for study has been selected, the research proposal should be discussed with appropriate health officials. Through their assistance or approval, contact is then made with key individuals in the sampling area. A community leader, public health nurse, or perhaps an influential housewife can insure a successful serum survey by assisting in making community contacts. During an epidemic, coverage by the news media may be helpful. A newspaper picture of the survey team which can be shown to persons who suspect ulterior motives will allay their fears and aid in obtaining their cooperation.

### Selection of Subjects to Bleed

Serological studies are most frequently directed toward specific portions of the human population which can be divided into general categories as follows:

1. Clinical cases exhibiting fever with or without specific symptoms of arbovirus infection.
2. Family contacts of cases.
3. Persons without illness but suspected of being at high risk, such as woodsmen, fishermen, field construction workers, farmers and forest rangers.
4. Persons residing under certain environmental conditions being studied, such as rural, urban, high or low socio-economic level census tracts, etc.

Blood specimens from the indigenous population are more significant than those from transients, since the latter may confuse data interpretation. The number of individuals to be sampled should be considered carefully, preferably with the consultation of a statistician. Arbitrarily, at least 80-100 blood specimens comprising at least three different age groups should be collected for each category. Example of age groups are: 0-14, 15-49, 50+, with a distribution of approximately 30% under the age of 15 and 20% over the age of 50. It is important to sample different age groups in order to make inferences regarding time relationships of arbovirus activity within particular areas.

### The Survey Team

An effective survey team consists of a physician and/or a nurse to interview the subject and draw the blood specimen, and an assistant, often a para-medical person well known in the area, to record the data and prepare labels for the specimen tubes.

Arbovirus Serological Survey - March 1969
Georgia Health Department in Cooperation with the National CDC

6

Head of Household: CULPEPPER, (Last) EBENEZER (BEN) (First) Q (Middle Initial)

Date March 6, 1969

Street Address: 40 SOUTH MAIN, MACON

Apt. No. 2

Telephone Number: 427-0143

Census Tract 12

| All Persons in Household First names, middle initials | Age Yrs. | Sex M | Sex F | Birth Place City | Birth Place State | *Length of Residence at Present Locale | *Length of Residence at County | * Mil. Serv. | Y F | * For'gn Trav | Lab Control Numbers Field Number | Lab Number | Laboratory Findings |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. BEN Q. | 47 | ✓ | | DALLAS | TEX. | 17 yrs. | 17 yrs. | ✓ | ✓ | ✓ | 237 | P 26041 | YF HI 1:20 CF <1:8 |
| 2. MARY R. | 40 | | ✓ | HOUSTON | TEX. | 17 yrs. | 17 yrs. | – | – | ✓ | 238 | P 26042 | SLE HI 1:40 CF <1:8 |
| 3. ROBERT E. | 16 | ✓ | | MACON | GA. | 16 yrs. | 16 yrs. | – | – | | 239 | P 26043 | NEG |
| 4. DONNA D. | 12 | | ✓ | MACON | GA. | 12 yrs. | 12 yrs. | – | – | | 240 | P 26044 | NEG |
| 5. | | | | | | | | | | | | | |
| 6. | | | | | | | | | | | | | |
| 7. | | | | | | | | | | | | | |
| 8. | | | | | | | | | | | | | |

**Data on Household Head**

Occupation:
1. executive
2. housewife
3,4 student

Avocation:
camping
bird watching

Education:
(1)__ Post Graduate
(2)✓ College Graduate
(3)__ College 1-3 Years
(4)__ High School Graduate
(5)__ 10-11 Years
(6)__ 7-9 Years
(7)__ 0-6 Years
(8)__ Unknown

Ethnic Group:
(1)✓ Caucasian
(2)__ Negro
(3)__ Other

INTERVIEW COMPLETED ☑
INTERVIEW NOT COMPLETED ☐
(1)__ Not-at-Home
(2)__ Vacant Unit
(3)__ Refusal
(4)__ Other, specify_____

Coding Symbols: Yes - ☑ No - ☐ Unknown ?

Interview Sector Number 24

Housing Unit Number 3

Team No. 1

* Complete reverse side.

7

## Residence History

| Person Number | Street Address; if other city, give name | State | Dates From | Dates To |
|---|---|---|---|---|
| 1 | 272 E. Texas Blvd. Dallas | Texas | 1921 | 1928 |
| " | 4810 Ponce de Leon Atlanta | GA. | 1928 | 1951 |
| " | present | | 1951 | 1968 |
| 2 | 430 Parkway Drive Houston | Texas | 1928 | 1951 |
| " | present | | 1951 | 1968 |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

| Person Number | History of Recent Illness: Fever | Headache | Lethargy | Rash | Myalgia | Diarrhea | Respiratory | Other Symptoms and Duration | Date |
|---|---|---|---|---|---|---|---|---|---|
| 4 | ✓ | ✓ | | | ✓ | | ✓ | 5 days | June |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |

Presence of Wild or Domestic Vertebrates

several bird feeders near house.

2 dogs (pets) at home

**Military Service**

| Person No. | Geographic Area | Dates From | Dates To |
|---|---|---|---|
| 1 | So. Pacific | 1943 | 1944 |
| | | | |

**Foreign Travel**

| Person No. | Geographic Area | Dates From | Dates To |
|---|---|---|---|
| 1,2 | Europe | July 1961 | Sept 1961 |
| | | | |
| | | | |

**Lab Results**

| | HI | EE | WE | SLE | Calif | YF | D-II |
|---|---|---|---|---|---|---|---|
| P26041 | <10 | <10 | <10 | <10 | 20 | <10 | |
| P26042 | <10 | <10 | 40 | <10 | <10 | <10 | |
| CF | | | | | | | |
| P26041 | N.D. | N.D. | N.D. | N.D. | <8 | ND | |
| P26042 | N.D. | N.D. | <8 | N.D. | ND | ND. | |

the type of work the household head does, not to the name of the concern or the place of employment. If the household head is retired, enter his previous occupation and current status on the form, e.g., retired — carpenter.

**Antibody Studies**

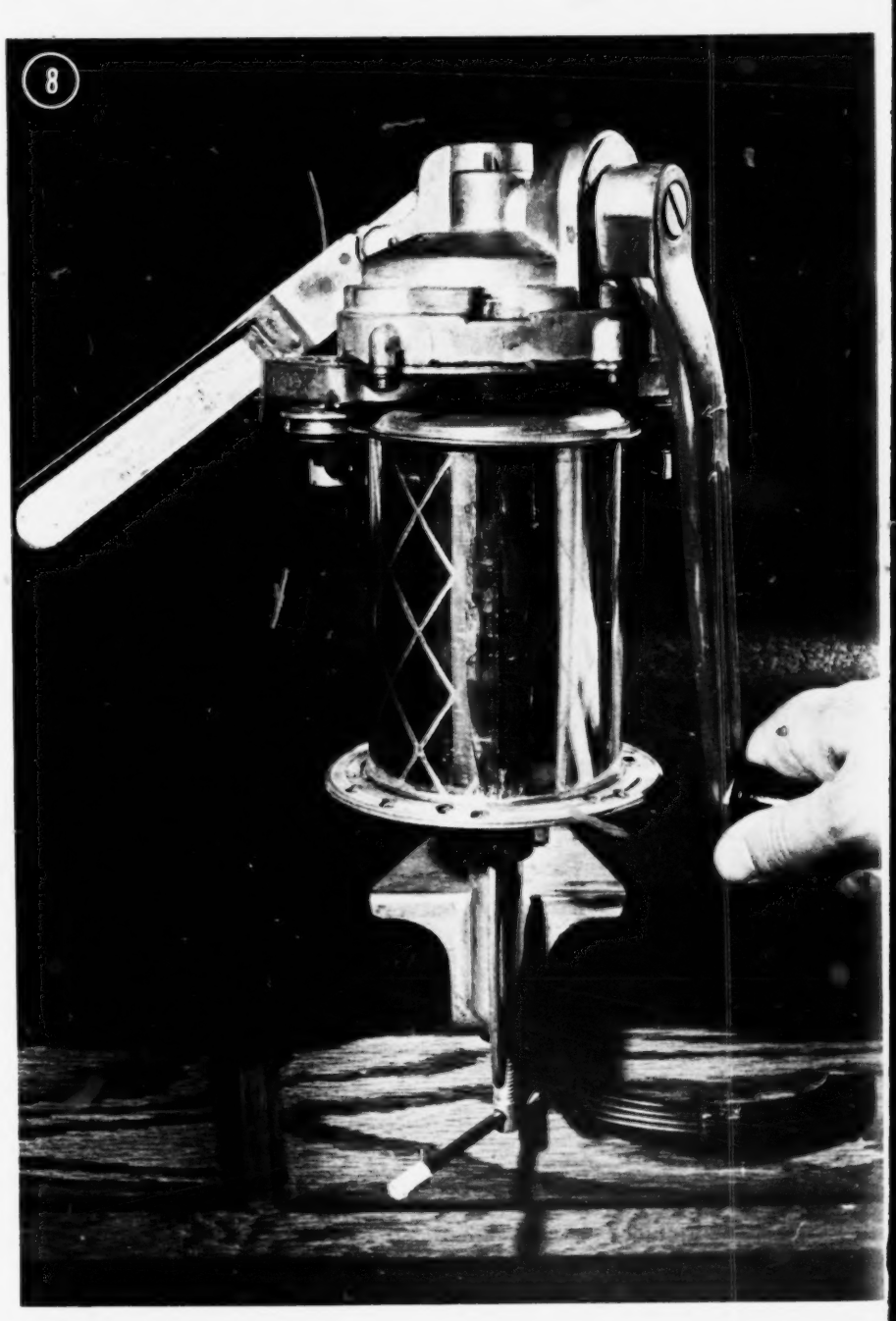
Specimens collected for antibody studies need not be refrigerated during the day except in extremely hot climates, but they should be kept out of direct sunlight. Maximum clot retraction occurs if the clot is rimmed with a sterile applicator stick and refrigerated at 4°C overnight. Use aseptic techniques to avoid contamination which may cause anticomplementary activity. Centrifuge for 10 minutes at 1000 rpm, and pour the serum off into two dram screw-cap vials and store at —20°C. At every step of serum transfer, the adhesive label must be transferred to the new tube to avoid labeling errors. Do not freeze whole blood since hemolysis precludes accurate analysis by serological tests.

When facilities in the field are not adequate for processing the serum, specimens of whole blood can be shipped without refrigeration to the diagnostic laboratory provided that no more than a day or two is required for transit. This, of course, applies only to samples collected for antibody studies.

**Virus Isolation Studies**

Except for autopsy material, blood has been the most productive source for recovery of arboviruses from human cases. Isolations from cerebrospinal fluid are uncommon and generally occur in association with viremia. The viremia may occur prior to fever or be associated with it; therefore, it is absolutely essential that blood samples for virus isolation be taken as soon as the febrile patient is seen. Take 10 to 20 ml of blood, allow it to clot, centrifuge at 1000 rpm for 10 minutes and decant the serum. Store both the serum and clot at —70°C temperature immediately, either on dry ice or in a mechanical freezer until shipped by the fastest method to the diagnostic laboratory. If dry ice is used, an effort should be made to keep the $CO_2$ from penetrating the sample since a lowering of pH is deleterious to arboviruses.

Adequate *temporary* sealing for storage on dry ice can be accomplished by placing the tightly stoppered serum tubes in a plastic bag which is securely fastened and placed in a container. Carbon dioxide acidification can virtually be eliminated if the tubes are sealed in metal cans using a home can sealer available at hardware stores or from mail order houses (**Figure 8**). The cans also reduce the chance of breakage.



For shipment, use an insulated shipping container with enough dry ice to last at least two days in case of an unexpected delay in delivery. Approximately 20-25 pounds of dry ice is adequate for a two-cubic-foot styrofoam shipping container. The receiving laboratory should be notified by wire or telephone of the method of shipment, the carrier and flight number, the way bill number, and the expected time of arrival.

If autopsy material is available, several different sections of each organ to be tested should be placed in specimen jars or plastic bags, frozen immediately at —70°C, and transported to the laboratory on dry ice in a well insulated shipping container for virus studies. Additional portions of each organ should also be fixed in formalin for histopathological studies.

## FARM ANIMAL SPECIMENS

### General Considerations

Farm animals may be important resources to confirm current virus activity by virus isolation tests, or to reveal extent of past virus activity by antibody studies. If clinical illness compatible with arbovirus infection is evident in the farm animals, virus isolations should be attempted from the blood, or if animals die, from the brain. Studies with eastern encephalitis (EE) and western encephalitis (WE) have shown that at the time of the animal's death the viremia usually has disappeared; however, the brain may still contain virus.

Prior to any studies with farm animals, permission must be obtained from the owner. Obtaining this permission frequently requires a degree of salesmanship which is often aided by briefly explaining to the owner the purpose and public health value of the study and what will be done to the animal.

The study plan should include the overall number of specimens to be taken and the number desired from each farm site. Information is required on the age and type of animal, the length of time each animal has been in the area, and any immunizations received.

### Assistance of Local Veterinarians

Consulting with local veterinarians and obtaining their cooperation is invaluable in studies of farm animals. They are usually aware of past or current arbovirus activity in the area and can help to procure the blood or tissue specimens. They may provide information about the location of cases and indicate which of the farmers might be the most cooperative.

### Handling and Restraint of Animals

When possible, work with farm animals should be done by veterinarians who are familiar with handling them. If a veterinarian is not available, use only trained personnel. Many of the animals are of economic or sentimental value and poor technique in handling them may be disastrous to public relations. In the event of injury or death due to handling, the question of liability must be considered.

Bleeding large animals is hazardous since they bite and kick. A rope halter may be sufficient restraint when working with horses, if the owner helps to quiet the animal. A chute may be used to restrain cattle, but should be used with caution for horses since they may panic and injure themselves.

Small pigs are turned over on their backs and held in position for bleeding. For large pigs, a hog snare or lariat usually is required for restraint.

Additional restraining techniques may be found in the book *Restraint of Animals* by Leahy and Barrows (3) which is available from the Cornell Campus Store, Inc., Ithaca, New York 14850.

### Bleeding Techniques

The external jugular vein is used to bleed horses and cattle. An area over the vein is disinfected by scrubbing with 70% alcohol. Finger pressure is applied to block the jugular vein distal to the head. An 18 gauge, 1½" needle on a 20 ml syringe is inserted into the raised vein at an angle, and slight negative

pressure applied to the syringe barrel (**Figure 9**). Evacuated glass tubes (Vacutainers) may be used instead of syringes and needles. About 20 ml of blood is withdrawn. Then the finger pressure is released, a clean, dry cotton pledget placed over the insertion point of the needle and pressure applied on the pledget as the needle is removed from the vein.

Large samples of blood (10-20 ml) from pigs are most satisfactorily taken from the anterior vena cava. A 20 ml syringe with a 6″, 18 gauge needle is used for adult hogs; a 2½″, 20 gauge needle is adequate for a pig 40 pounds or under. From a position in front of the pig, insert the needle at the midplane at the base of the throat above the anterior sternum. The needle is directed toward the base of the tail. Apply slight negative pressure on the barrel; presence of blood in the syringe will indicate when the vena cava is reached.

The ear or caudal vein may be used if only a small amount of blood is needed. A 20-24 gauge, 1″ needle on a 1 or 2 ml syringe can be used.

### Handling of Blood Samples and Tissues

For virus isolation and antibody studies, blood samples from farm animals should be handled in the same manner as those from humans, as previously described.

Brain is the tissue of choice to attempt isolation of virus from horses presumed to have died of encephalitis. The removal of the horse brain is best left to a veterinarian since specialized surgical tools are required. Amateurish attempts without the proper tools should be avoided.

The veterinarian should remove the entire brain, or at least an intact half from either side. Portions of each major part of the brain should be preserved in formalin for histopathological studies, and other portions placed in separate labeled containers and frozen on dry ice for shipment to the laboratory for virus studies as described for human autopsy material.

## WILD MAMMAL SPECIMENS

### Permit Requirements

All states require a permit to take wild mammals for scientific study. These permits are obtained by writing to the appropriate state agency for application forms. Information is required about the nature of the study, numbers and types of animals to be taken, capture methods to be used, and a list of persons who will perform the work. The completed application form should be submitted by the person responsible for the study. A yearly report which lists the numbers of each species taken and the disposition of the animals killed is usually required.

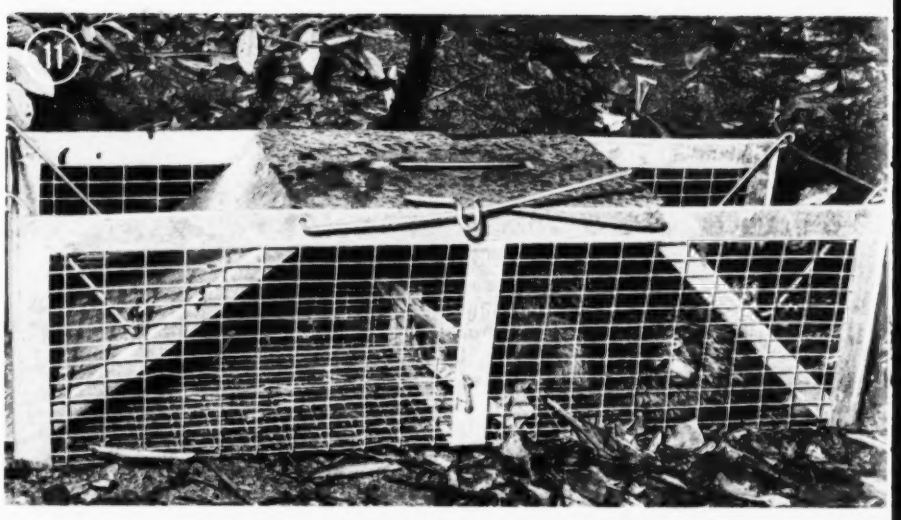
### Types of Mammal Traps

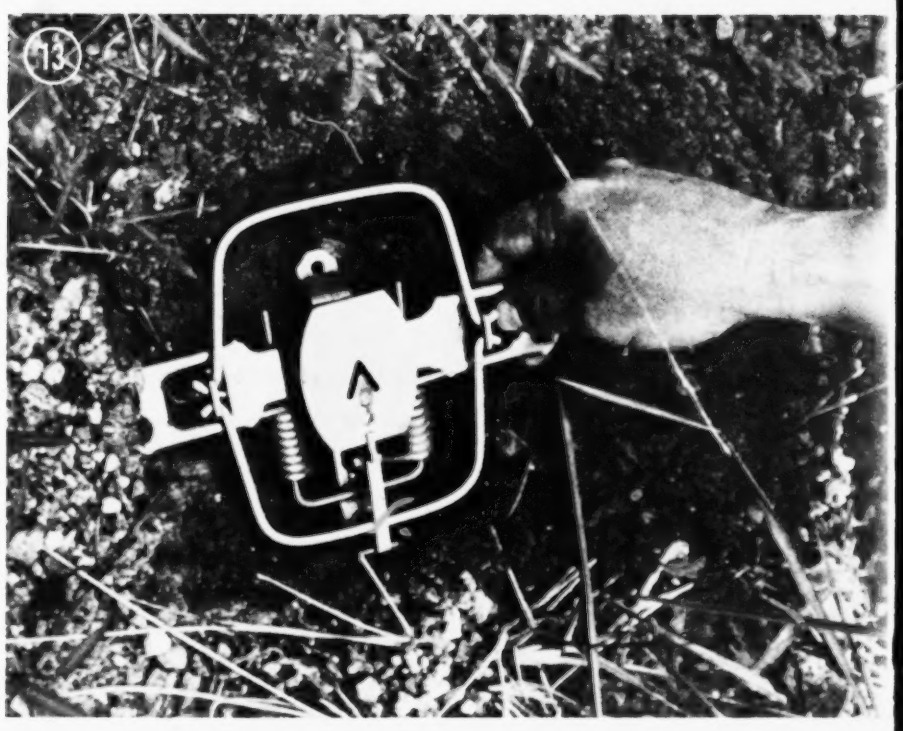
Arbovirus-vertebrate studies generally are planned so that wild mammals are captured, marked for identification, and then released unharmed after a blood sample is obtained. A variety of live traps are available for this purpose. A list of trap types, sizes and approximate cost, and the names and addresses of several suppliers are provided in the Appendix.

The Sherman type trap is a rectangular metal box with a trap door at one end which is simple to set (Figure 10). It is available in various sizes and materials and in either collapsible or non-collapsible models. The 3"x3"x 9" trap constructed of 28 gauge metal is particularly versatile.

The Havahart type trap is constructed of wire except for a solid metal roof; it has trap doors at both ends (**Figure 11**). Sizes number 2 and 3 are especially useful but somewhat difficult to carry or ship in numbers because they are not collapsible.

National type traps are constructed of wire throughout, with a trap door at one end (**Figure 12**). This trap is collapsible, an obvious advantage for shipping.






Steel traps are most frequently used to capture large predatory animals such as foxes, raccoons, and bobcats (**Figure 13**). It is recommended that these traps be used only when other types of live traps prove inadequate.

Wooden box traps are not available commercially; however, plans for them may be found in the manual, *Wildlife Investigational Techniques* (4). Wooden box traps are slightly superior to metal traps in the winter when they provide a refuge from the elements. Otherwise, they are bulky, heavy and usually suitable only for permanent study areas.

An oil can snap trap may be made from regular mouse or rat snap traps coupled to No. 10 oil cans (**Figure 14**). A plan for building these traps may be found in a manual by Davis (5) published in 1956. Unfortunately, this manual is out of print, but it may still be available in some libraries.

**Selection of Bait**

Selection of bait may be crucial to the success of the investigation, depending upon the season and species desired. In most arbovirus investigations, the predominant wild mammals are usually the ones desired because of their numbers and possible implication in epizootics. In temperate zones rodents are generally the most abundant mammals. Rodents can be classified into two main groups according to feeding habits: the seed eaters or seminivorous rodents, and the grass eaters or herbivorous rodents. Usually both seminivorous and herbivorous rodents will be attracted to peanut butter bait (**Figure 15**). A good bait mixture is peanut butter with rolled oats. Crunchy nut peanut butter is recommended in areas where ants are abundant. Another good bait is a mixture of beef tallow and chicken feed. Herbivorous rodents are also attracted to apples, prunes and carrots.

Most rabbit species may be baited with apples and carrots or corn in the winter. Another method is to pre-bait the area by scattering commercial rabbit pellets and then later use this same food as bait in the rabbit traps. Some rodents feed upon insects at certain times of the year; therefore, possibly some bait with an insect base might prove satisfactory.

The most effective bait for trapping predators with steel traps is urine. Urine baits can be obtained by placing an animal in a cage with a wire floor and a piece of galvanized metal underneath so shaped as to catch and funnel all urine into a container. The amount of urine bait required is approximately one to two tablespoons at each trap site. Wildcat urine is recommended; however, other animal urine may be used.

**Field Trapping Plans**

We have found a two-man trapping team to be highly effective. Effective collecting of mammals is, in part, a matter of simple arithmetic. For example, if 100 small mammal blood samples are desired in five days and the rate of capture is 10 per 100 trap nights, obviously at least 200 traps should be set each night. This, hopefully, should maintain the 20 per day average capture rate necessary to meet the original objective. There is no way to estimate adequately the number of animals that can be captured in an area at any time. In temperate zones the average is approximately one animal for every 7-10 trap nights. Annual cycles of abundance are pronounced in rodents. The late winter low may be as much as 20-fold less than the late summer peak. However, annual peaks and lows of population fluctuations may not necessarily be accurately reflected in trapping success since bait attraction may vary with the season.

The number of traps that two men can set in a day varies with the type of trap, the type of terrain, and the survey method used. A preferred survey method is a line-transect carried out along the edge of a road which bisects the principal habitats of an area. This method permits the placement of the greatest number of traps in the shortest possible time, e.g., in one day two workers can place 50 to 70 steel traps spaced every 1/10 of a mile along a primitive road. They can similarly place as many as 200 National or Havahart traps or as many as 400 Sherman traps. Usually a combination of these traps is used, and the time requirements will vary accordingly.

Another good system is to place one steel trap, one Havahart or National trap, and two Sherman traps per station, separating each station by 1/10 of a mile. In this arrangement, two workers can set about 50 or 60 stations a day. The number of stations that can be set on subsequent days is dependent upon the number of animals captured and the time required to process them. This is a self-regulating system wherein sparse populations receive more traps and dense populations fewer traps, resulting in a nearly optimum number of traps per day in either case.

**Trap Placement**

The spacing of traps along roads at some predetermined interval such as 1/10 of a mile is an efficient way to cover a long traverse. However, the manner of placement of traps at any station can affect the success of capture. Fallen trees (**Figure 16**), rocks (**Figure 17**), and other debris are attractive to rodents and traps placed nearby usually have greater success than those placed in open areas lacking cover. Frequently traps are vandalized; therefore, they should be hidden under vegetative cover when possible. This has the added advantage of providing shade during hot weather.

A line of steel traps is most effective along dirt roads or streams. A shallow depression is scraped out of the earth to match the shape of the trap. The trap is placed in the depression and a cloth patch placed over the pan to prevent dirt from sifting under the pan which would prevent its being triggered (**Figure 18**). The trap is then covered smoothly with dirt (**Figure 19**). A drag is attached to the trap (**Figure 20**) and about two tablespoonfuls of urine are placed near the pan as bait.

**Trap Inspection and Sample Size**

Normally the traps are visited once a day, as early as possible in the morning. In the case of very dense populations and trapping of diurnal mammals such as squirrels, traps should be visited at least twice a day—morning and evening—and occasionally during the middle of the day. Traps should be operated for at least three days but preferably for not more than seven days in one location. The first three days' trapping usually yields about 75% of the number of mammals that will be taken in the area.

Determination of what constitutes an adequate sample of animals is, in part, a statistical problem. Davis and Zippin (6) present tables for selecting sample size based on specified significance levels and the size of error allowed by the investigator. In surveys for presence or absence of arbovirus activity in mammals, the authors have arbitrarily chosen a sample size of between 100 and 200 individuals.

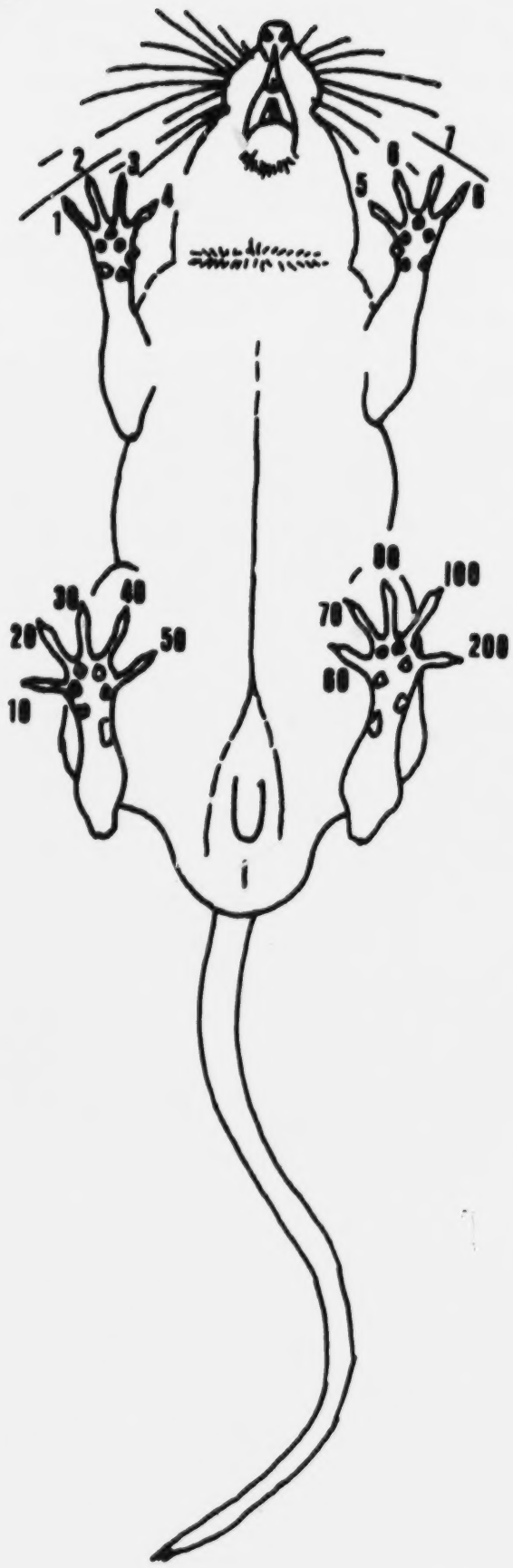
**Identification, Age Determination, and Marking**

Some useful books on identification of mammals in the United States have been published (7-10). Identification of most mammals from these books is relatively simple. Some rodents, however, are particularly difficult to identify and may require verification by a competent taxonomist. This may be done by shipping prepared study skins and associated skulls, or the frozen or formaldehyde preserved carcasses, to museum specialists. Therefore, care should be taken when dissecting animals to obtain tissues for virus tests not to mutilate the skull and other diagnostic features required for identification.

Determination of animal age is important to the arbovirologist because it may indicate the period of virus activity. Techniques of age determination for different mammals vary so that references should be consulted for each species. Some larger animals may be aged by counting cementum layers in teeth. The age of some species can be estimated from the dry weight of the lens of the eye if the growth rate of the lens is known. Degree of closure of the epiphyses of long bones permits detection of juvenile mammals for some time after they have achieved adult size. Pelage characteristics may be used in separating juvenile cottontail rabbits and gray foxes from adults.

Mammals can be marked in a variety of ways. Metal fish tags of various sizes available from the National Band and Tag Company, Newport, Kentucky 41071, can be used as ear tags. Mammals with unpigmented ear skin can be permanently tattooed using a tattoo kit available from the same company. Systems of toe clipping are useful in permanently marking animals (**Figure 21**). Occasionally when small numbers of animals are to be marked, ear notching or cropping may be applied. However, the scissors or bone forceps used for toe clipping and ear notching or needles used for tattooing may carry virus from animal to animal; therefore, all such equipment must be sterilized between each marking.

21



**Animal Handling and Restraint**

Marking and bleeding are usually done at the capture site. However, it may be more efficient to take the animals to a field laboratory for bleeding, weighing, measuring, and dissection of tissues. The animals can be carried in the traps in which they were caught, with each trap labeled as to site of capture. Each trap removed should be replaced with a new trap to save doubling back.

There is risk of injury in handling wild mammals but common sense and the proper use of anesthesia will minimize this risk. Hazardous anesthetics should be used with proper precautions to avoid injury to the collector or the animal. Ether, for example, is effective but the hazards of fire are great and working in a poorly ventilated area must be avoided; also, critical judgment is required in its use since overdosage may kill the animal.

Tranquilizer guns using a variety of drugs are available. These have proven useful for large animals, but dosages are critical and have not been determined adequately for small animals. Further experimentation will be required before we can recommend tranquilizing guns for general use.

Recently, personnel in the Arbovirus Ecology Laboratory (NCDC) have adopted $CO_2$ as an anesthesia. A simple anesthetizing chamber is made by dropping small chunks of dry ice into a gallon-sized plastic container (**Figure 22**). The animal is shaken from the trap into the $CO_2$ atmosphere in the container (**Figure 23**) and prevented from jumping out by quickly replacing the lid until the animal is anesthetized (**Figure 24**). In only a *few seconds*, the animal is unconscious and may be bled (**Figure 25**). Again, the dosage is critical; too much exposure to $CO_2$ will kill the animal and too little may permit it to recover before the bleeding is finished. As a precaution, the animal should always be

gripped firmly during the bleeding procedures and quickly released after bleeding. The action of $CO_2$ is unique because the animal is knocked down rapidly but after a short period of quiesence, recovers equally as rapidly. As with all anesthetics, some animals may be lost until one learns to judge the time of exposure. In the event of overexposure, artificial resuscitation may aid the animal's recovery.

A pair of heavy gloves should be worn when removing larger mammals such as rabbits and opossums from National traps. Rabbits may be grasped by the waist and withdrawn from the trap. Opossums are best pulled out by the tail. With more aggressive mammals such as raccoons, weasels, and particularly skunks because of their likelihood of spraying, it is safer to place the trap still containing the animal in a large chamber such as a garbage can and introduce the anesthesia into the can. Gentle handling of skunks usually will avoid their spraying. Taking aggressive mammals from steel traps is facilitated by restraining them with a choke stick and inoculating them intraperitoneally with Nembutal, approximately 1 ml for every five pounds of body weight. Some experimentation may be necessary to judge the exact dosage required. Frequently 20-30 minutes are required for the anesthesia to take effect. The animals should be placed in the shade during the waiting period. When anesthetized, the animal can be labeled by tying a data tag to one leg. To transport the animal to the field laboratory, place it in a burlap bag in a shaded, well-ventilated spot in the field vehicle.

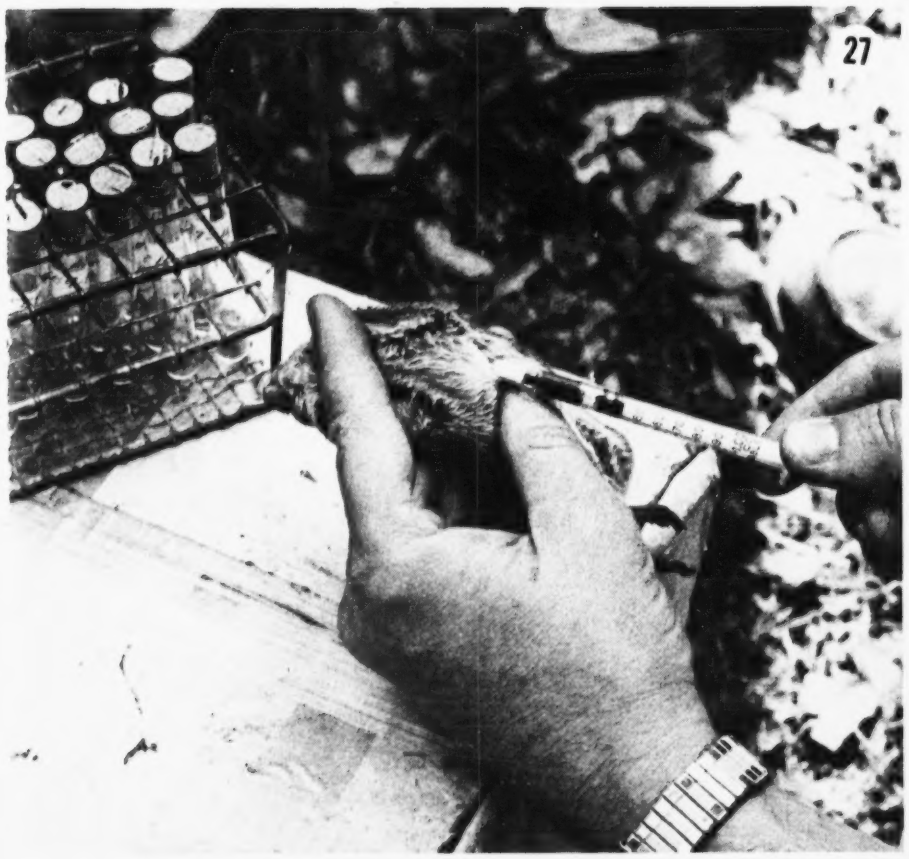
**Amounts of Blood Required**

When possible, obtain 2 to 4 ml of blood from each animals so that sufficient undiluted serum will be available for a variety of laboratory tests. From many of the smaller mammals, however, it is impossible to obtain such large amounts and some dilution will be necessary. To avoid confusion, only two diluting factors are generally used: when 0.1 ml to 0.4 ml of blood is taken, a 1 + 4 dilution is made; with 0.5 ml to 1.9 ml of blood, a 1 + 1 dilution is made. For ease in laboratory handling, we have standardized on a 0.2 ml volume of blood from small mammals and add 0.8 ml of diluent. It is generally more efficient to pre-dispense the proper amount of diluent into tubes and also to affix blank adhesive tape labels to them before going into the field (**Figure 26**).

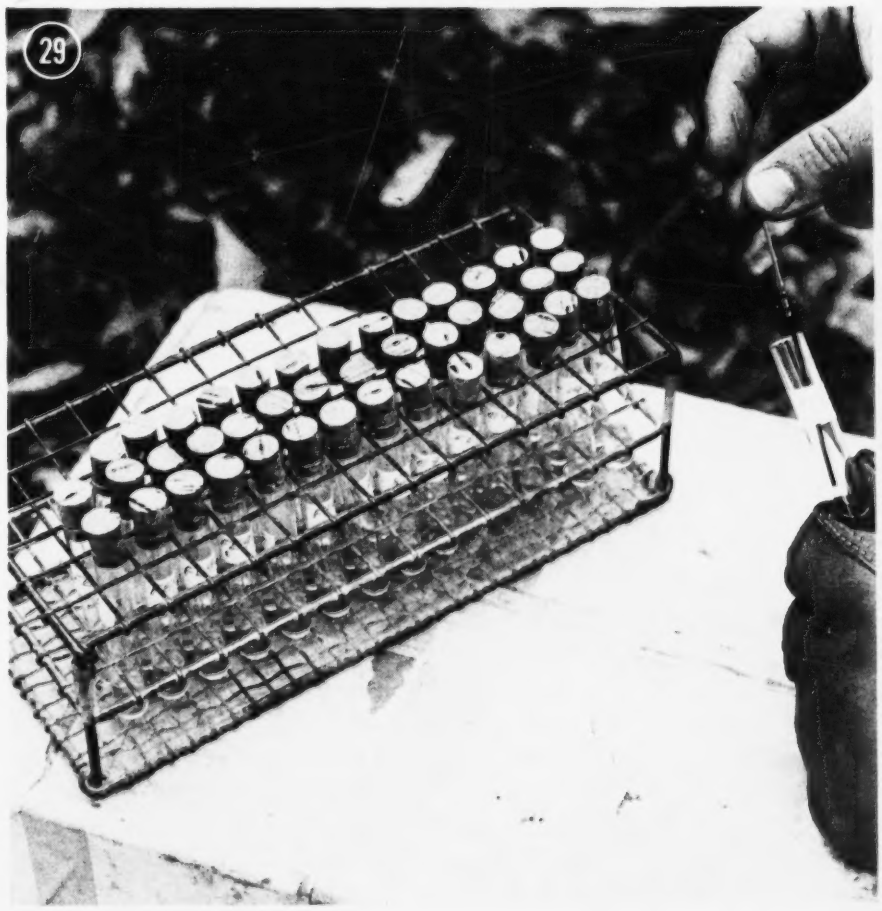
**Bleeding Techniques**

Anesthetized wild mammals may be bled from the heart (**Figure 27**). Disposable needles and syringes are recommended. The appropriate needle size is dependent upon the size of the animal. For small mammals the size of field mice, use a 25 gauge ⅜″ to ⅝″ needle; for mammals up to the size of rabbits, a 23 gauge 1″ needle; and for mammals the size of foxes and raccoons, an 18 gauge 1½″ needle. Either a 2 or 5 ml syringe is usually satisfactory.

The mortality risk associated with cardiac puncture can be avoided by bleeding from the brachial or inguinal veins. Needles no larger than 25 gauge are recommended for these veins.




We prefer to take the blood sample from the orbital sinus of small rodents and bats. The rodent or bat is anesthetized and held firmly in the left hand, with the thumb exerting sufficient tension just behind the eye to cause the eye to bulge out slightly. Either a 50 or 100 microliter micro-sampling pipet (see Appendix, page 64) is inserted into the posterior corner of the eye and gently rotated so that the capillaries are ruptured against the bone (**Figure 28**). The 50 microliter pipet will take up 0.1 ml of blood, the 100 microliter pipet, 0.2 ml. The pipet is then discharged into a tube containing a measured volume of diluent (**Figure 29**). Small capillary pipet bulbs can be used to discharge the samples. Apparently the animal is not seriously harmed; such bleedings may be continued daily for up to 10-15 days if necessary. This technique should not be used on birds.




**Diluent and Antibiotics**

The diluent generally used at the NCDC is 25% normal rabbit serum in phosphate buffered saline, pH 7.6 — 7.8, containing 1.6 mg streptomycin sulfate and 1000 units of sodium penicillin G per ml. The formula for the preparation of this diluent is given in the Appendix, page 65.

**Serum Extraction and Storage**

A small 6-place table model AC centrifuge is convenient for field work. If line current is not available, it can be operated from

a portable generator (**Figure 30**). A supplier of such a generator is listed in the Appendix, page 65

Serum extraction and storage procedures are as already described on page 11.

**Dissection and Tissue Collection**

Occasionally it will be impossible to obtain a blood specimen from an animal. In such cases, the heart, brain or some other tissue may be dissected from the animal and used for virus isolation attempts. Also, special studies may require that animals be purposely sacrificed so that a number of different tissues can be examined for virus: brain, salivary gland, heart, lung, liver, kidney, spleen, testis or ovary, and mammary tissue from lactating females.

Field dissection equipment includes forceps, disposable scalpels or single-edged razor blades, scissors, bone shears, and bone saws. An ordinary hack saw will serve as a bone saw. The instruments should be sterilized by placing them in liquid disinfectant such as 2% Amphyl overnight. They should be washed and additionally sterilized in alcohol and flamed before reuse or boiled for 15 minutes. Adequate numbers of instruments should be available to use a separate set for each tissue. Approximately 1 gram of each tissue is adequate. Each tissue is placed in a separate vial and appropriately labeled. The vials containing tissues from a single animal are placed in a plastic bag, sealed, and stored at −70°C.

**Data Recording and Numbering Code**

Field records must be accurate if the data accumulated are to be interpreted correctly. Recording of field data is facilitated by the use of a record book or sheets designed to include all pertinent data (**Figure 31**). These sheets may be kept in a notebook or bound into booklet form. Danger of loss of valuable data is minimized if a carbon copy is made and kept apart from the original. A sample Field Record Sheet is shown in **Figure 32.**

Each blood sample taken should be assigned a different field number, and this number should not be used again. If an animal is recaptured, a new field number is assigned. This number is retained for each specimen throughout the entire laboratory processing operation. The field numbering system used by NCDC personnel consists of a combination of letters and numbers. For example, FEV8-27 may be identified as a blood sample taken in Florida, Everglades, Vertebrate, in the year 1968, from the 27th animal captured in that study. Thus, GWV7-88 would represent the 88th vertebrate blood sample taken in Waycross, Georgia, in 1967. The identity of the animal can be quickly determined by checking the field records.

FIELD RECORD

LOCATION: Mahogany Hammock Florida Everglades Nat'l Park COLLECTORS: Lord + Newhouse

32 TOTAL NETS/TRAPS: 175 Sherman 50 National

| DATE | FIELD NUMBER | BAND/TAG NUMBER | SPECIES | AGE/SEX | CAPT. | SITE | AMT. BL./DIL. | DIL. RATIO | SEROLOGY H.I. | SEROLOGY | ISOL. | MISC. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2/22/68 | FEV8-48 | toe clip T-21 | Peromyscus | A/M | N | Boardwalk | .2/.8 | | | | | |
| | 49 | T-22 | " | J/F | N | " | " | | | | | |
| | 50 | T-17 | Sigmodon | A/F | N | " | " | | | | | pregnant |
| | 51 | T-4 | Procyon | A/M | N | N-Trail | serum | | | | | |
| | 52 | ear tag CDC-283 | Sylvilagus | A/F | N | S-Trail | serum | | | | | lactating |
| | 53 | T-18 | Sigmodon | J/M | N | " | .2/.8 | | | | | |
| | 54 | T-23 | Peromyscus | J/F | N | " | " | | | | | |
| | 55 | T-11 | Didelphis | A/F | N | " | serum | | | | | 4 young in pouch |
| 2/23/68 | 56 | T-24 | Peromyscus | A/F | N | Boardwalk | .2/.8 | | | | | lactating |
| | 57 | T-25 | " | J/M | N | " | — | | | | | no sample escaped |
| | 58 | T-21 | " | A/M | R | " | .2/.8 | | | | | |
| | 59 | T-19 | Sigmodon | J/F | N | " | " | | | | | |
| | 60 | CDC-284 | Sylvilagus | J/F | N | N-Trail | serum | | | | | |
| | 61 | T-5 | Oryzomys | A/M | N | " | .2/.8 | | | | | |
| | 62 | T-4 | Procyon | A/M | R | " | serum | | | | | |
| | 63 | T-12 | Didelphis | J/M | N | S-Trail | serum | | | | | |
| | 64 | T-26 | Peromyscus | J/F | N | " | ~~.2/.8~~ | tube | broken in | | centrifuge | |
| | 65 | T-27 | " | J/F | N | " | .2/.8 | | | | | |
| | 66 | T-20 | Sigmodon | J/M | N | " | .2/.8 | | | | | |
| | 67 | T-5 | Procyon | A/F | N | " | serum | | | | | lactating |

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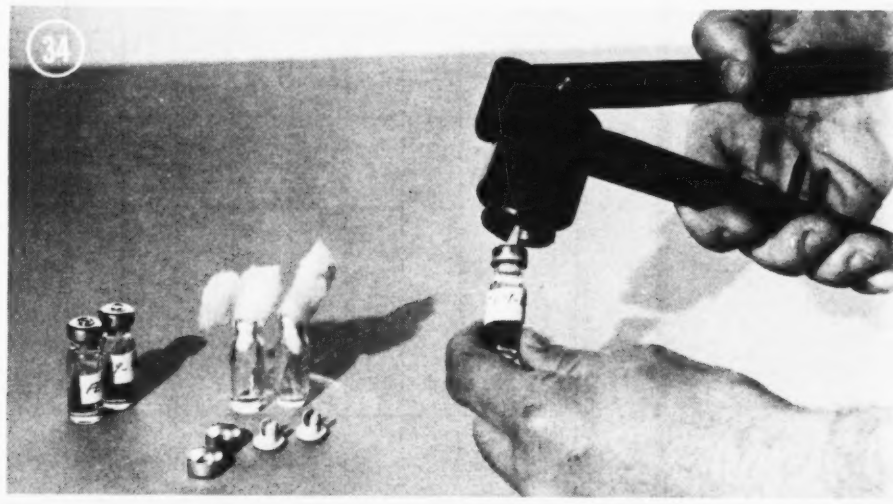
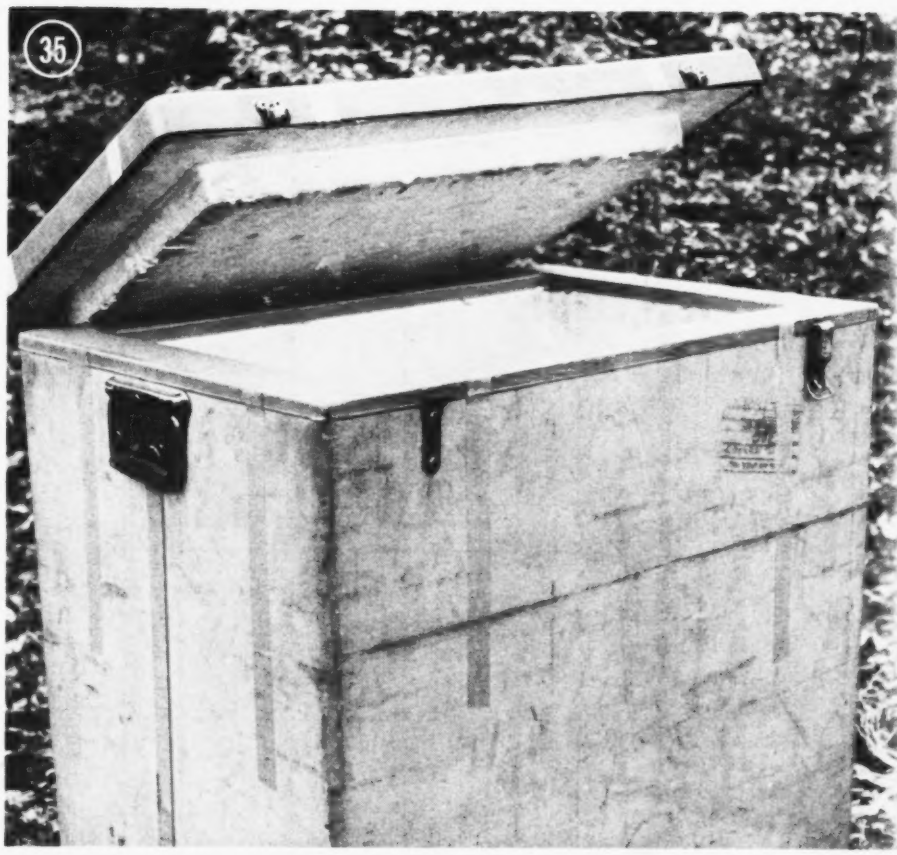
**Field Preservation and Shipping**

Blood samples taken in the field may be temporarily stored on wet ice (**Figure 33**). Increased serum yields are obtained by slanting the tube during clotting. The clotted blood samples are centrifuged and the serum or serum-diluent mixture drawn off, dispensed into vials, labeled, sealed, and frozen at −70°C. If only a small amount of serum is available and all must be reserved for antibody studies, the blood clot is frozen separately at −70°C and saved for the virus isolation tests.

When dry ice is used for storage, infiltration of $CO_2$ gas must be avoided since it can acidify the sample. Ordinary stoppers or screwcaps are not gas-tight at dry ice temperature; screwcap vials frequently loosen when frozen at −70°C, and rubber stoppers contract and cork stoppers are porous. One solution to the problem is the use of Wheaton bottles in which the stopper is held in place by a metal sealing ring (**Figure 34**). However, some stoppers are toxic to tissue cultures and these must be tested prior to use. The tubes accumulated during a day's work are placed in one plastic bag inside another. These bags are then sealed tightly before placing on dry ice. For more permanent protection against $CO_2$, the vials may be sealed in tin cans using a home canning device available from a mail order house.

A portable 5-cubic-foot chest with three-inch thick styrofoam walls is very useful for field storage (**Figure 35**). This chest holds 200 pounds of dry ice, and the insulation is so effective that usable amounts of dry ice remain for at least two weeks, even at summer temperatures. Dry ice preservation is enhanced by a one-inch layer of newspapers placed on top of the ice as added insulation; the chest lid should be removed only when necessary. See page 64 for supplier.





Smaller styrofoam shipping containers, 21″ x 13½″ x 20″, available from the same supplier, are useful in the field (**Figure 36**) or for transporting specimens to the laboratory. As a safety factor during shipping, sufficient dry ice should be added to keep the samples frozen two days beyond the expected time of arrival. The shipment should be sent by the fastest method; and the receiving laboratory notified of the carrier, flight number, waybill number, and expected time of arrival.

The use of liquid nitrogen for storage of specimens precludes the acidifying problem; however, the hazards of nitrogen spillage, explosion if the container is sealed, or the possibility of vials exploding when removed improperly from the container must be considered. Cost and availability may also be factors in its use; at present, there are only a limited number of suppliers of liquid nitrogen. When properly used, however, liquid nitrogen is excellent for storage of specimens.

Upon arrival at the laboratory, the specimens are stored in a —70°C mechanical freezer until the virus isolation tests are completed. Then the serum samples remaining can be transferred if desired to a —20°F freezer until serologically tested.

**Use of Sentinel Mammals**

Susceptible animals purposely exposed in the field to the bites of arbovirus vectors are referred to as "sentinel" animals.

Various animals may be used, the species depending upon the disease agent to be studied since some are more susceptible to one agent than to another. Examples of sentinel mammal species are hamsters, monkeys, suckling mice and rabbits. Various avian species such as chickens, pigeons and pheasants are also commonly used as sentinels and are discussed in a later section.

Sentinel animals require time and attention. They must be caged or penned in the field so that they are protected from the weather and predators but freely exposed to the bites of arthropod vectors. The confining unit should provide adequate food, shelter and security at minimum cost.

Important factors considered in selecting a sentinel mammal species are cost, ease of handling, its size and attractiveness to arthropod vectors, its age of greatest susceptibility, and its ability to withstand rigors of the climate. The design of the cage is critical and even the best types have limitations. Some species of mosquitoes are reluctant to fly through screening to reach an animal even when the size of mesh will allow it, or will not enter a shelter unless the light conditions or ventilation are particularly suitable. Finally, the field cage must permit ready access for the field workers since the animals must be checked frequently for signs of illness and for periodic bleeding. A sentinel rabbit cage used for study of the California virus complex (CE) by Dr. Wayne Thompson, University of Wisconsin, is shown in **Figure 37.**

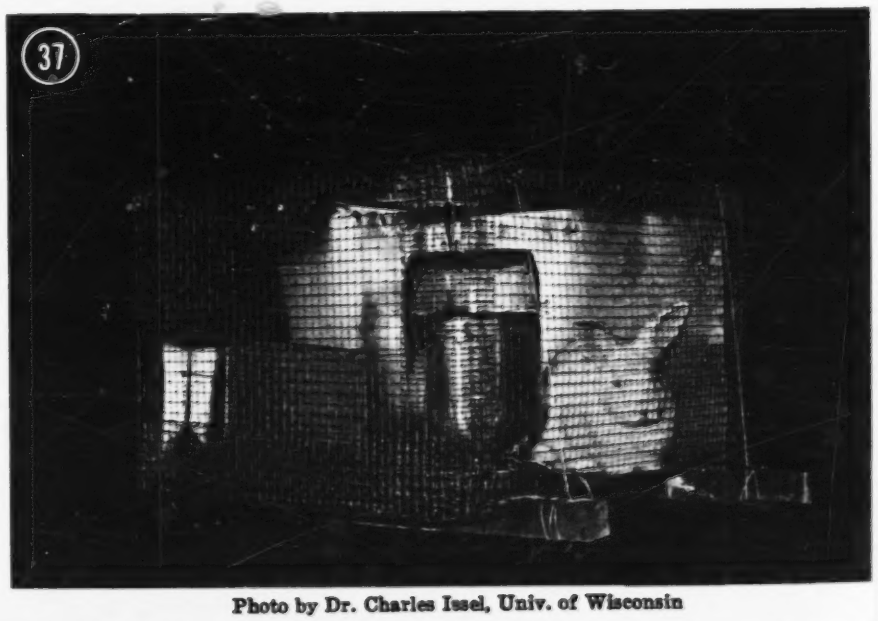



Photo by Dr. Charles Issel, Univ. of Wisconsin

Before use in the field, a sentinel animal is bled and tested to assure that it is non-immune to the virus being studied. At this time they are also banded or otherwise marked for subsequent identification. When placed in the field, a checking, bleeding, and testing schedule is established depending upon the goals of the study. Rabbits, for example, may be bled twice a week from the ear vein. The first sample each week is stored at —70°, and the second sample is serologically tested for presence of antibody. If antibody is found, the first sample is thawed and tested for virus. This system has been productive in California group arbovirus studies in the Midwest and Canada.

Hamsters can be exposed continuously for as long as desired; however, they should be examined daily and sick or dead animals taken to the laboratory for virus testing. In the absence of symptoms, the hamsters are bled at predetermined intervals as other sentinel animals. This species has been used successfully in VE studies in Central and South America.

Mosquitoes or other biting insects can be collected concurrently if the host shelters are designed to retain the arthropods attracted to the sentinels. Such devices described in entomological literature include sentinel mouse and hamster hoods and various cage, shed, and stable traps. By identifying, counting, and testing these insects, one can estimate certain vector population densities and infectivity rates.

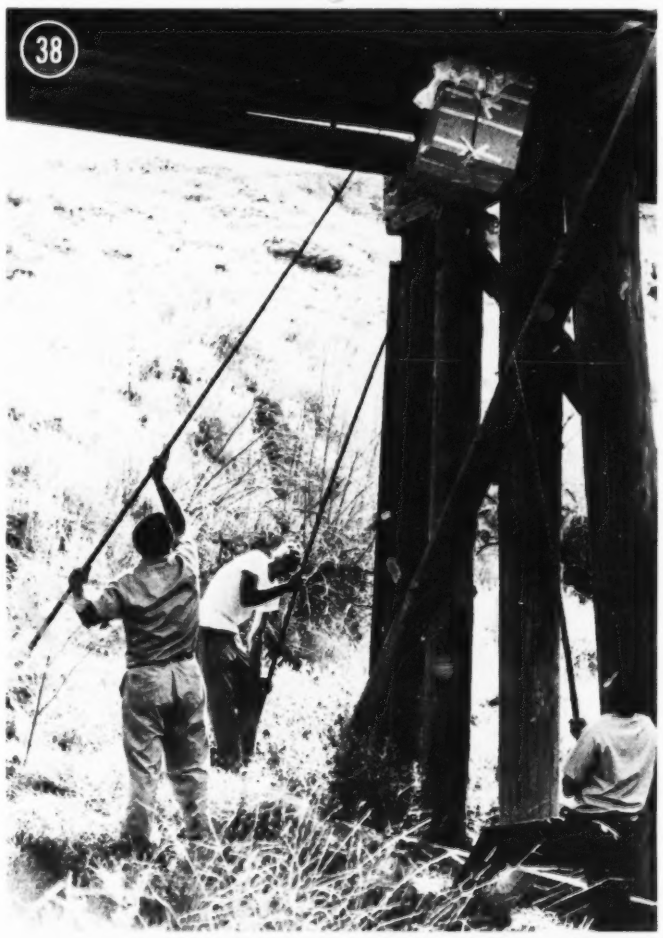
## BAT SPECIMENS

### General Considerations

Bats have been suspected as arbovirus hosts. Their importance, however, has not been adequately evaluated. Some bats migrate between the tropics and temperate zones and are thus potential transporters of arboviruses. Also, in the tropics, bats are probably the most abundant mammals; and if only because of their sheer numbers, warrant investigation to determine their role in arbovirus ecology. Since bats may be infected with rabies and rabies can be transmitted both by aerosols in bat caves and by bat bite, the worker should exercise caution and preferably should be immunized against rabies.

### Methods of Collection

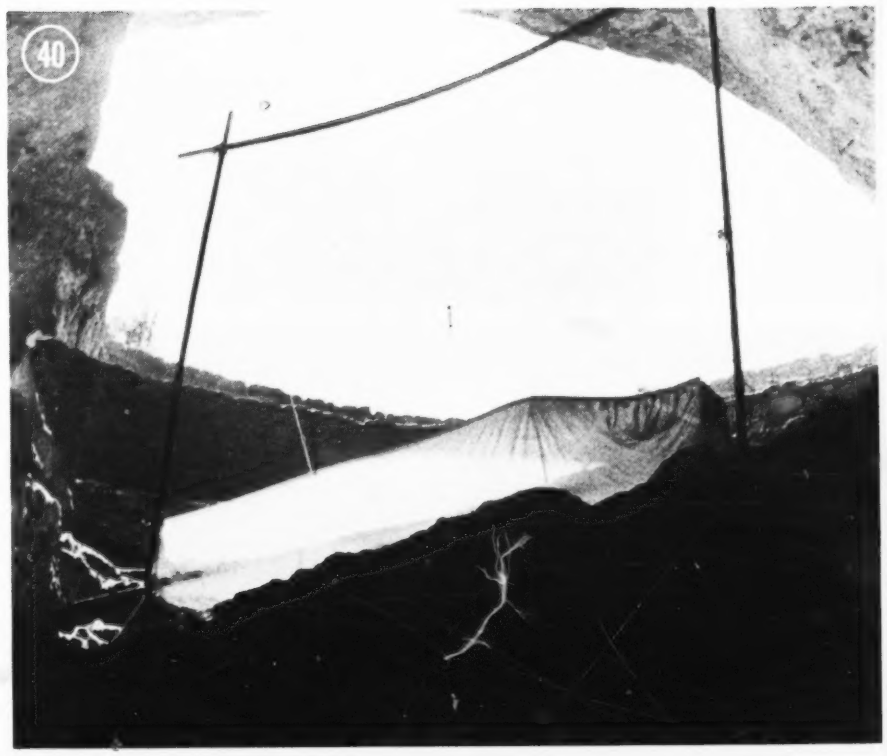
The habits of bats are only partially known; however, during the day most kinds of bats rest in dark secluded places such as caves, old buildings, crevices, underneath bridges, or in hollow trees. They may be dislodged from such places with a stick or stout wire (**Figure 38**) causing them to drop into large plastic bags (**Figure 39**).

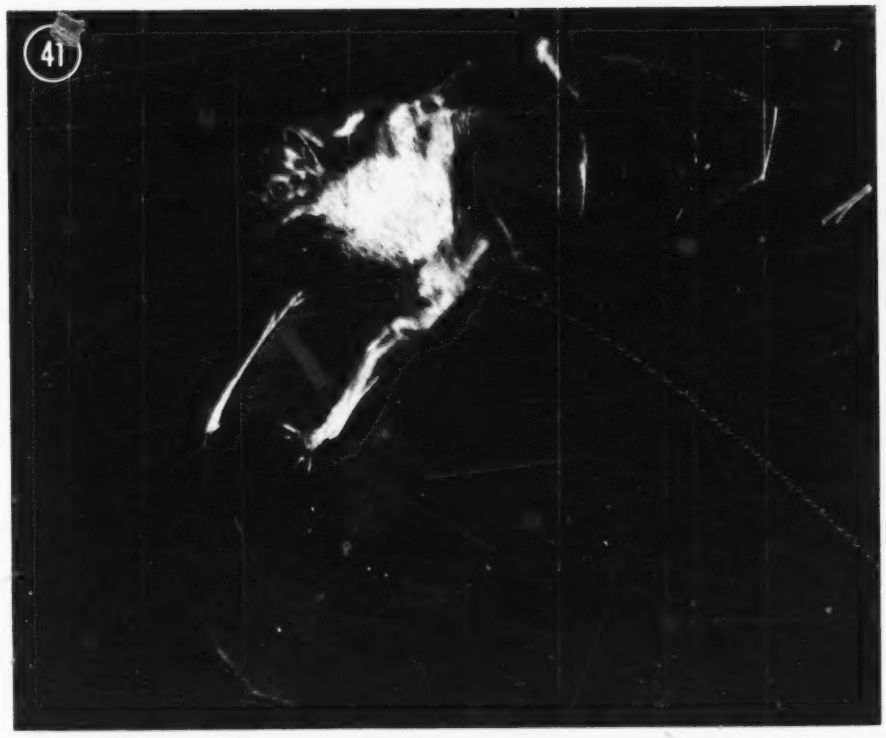


Bats become active at dusk and generally remain so throughout the first few hours of darkness. Some search out roosts later at night, to rest and digest their evening meal. Collections can also be made from these night roosts if their location is known. The bats become active again at dawn when they return to their day roosts. More studies are required to determine the amount of time bats spend in night roosts and their locations since night roosting may afford opportunity for potential arthropod vectors to feed upon them.

Mist nets, described in detail in the wild bird section on page 28 may be used to capture bats as well as birds. However, placement of the nets for bats is different. In dry areas, placement over available water is effective. Other good sites are along the tops of small hills devoid of vegetation, on the inner banks of streams or rivers near a sharp bend, and at the entrance to a bat cave or to a bat roost in an old building. The mist nets should be examined frequently during the night and the bats removed before they damage the net or chew their way to freedom. To remove a bat from a net, hold the bat with a gloved hand and use the other hand to cautiously untangle it. A bat can be anesthetized while still in the net to facilitate its removal by holding a wide-mouth jar containing ether or $CO_2$ against it from one side of the net and apply the jar lid from the other side.

Large numbers of cave-dwelling bats can be captured with the Constantine trap (11). This trap consists of a frame of piano wires strung vertically and a smooth-sided receptacle at the bottom to prevent the captured bats from escaping (**Figure 40**). When a bat flies into the piano wire, it is temporarily confused and slides or falls down the wire (**Figure 41**) into the collecting receptacle (**Figure 42**).



### Identification

The taxonomy of tropical bats is difficult and not well understood. However, most North American species can be identified through the use of the *Mammals of North America* (7). Bats that are difficult to identify can be preserved in formalin solution and sent to a bat taxonomist for identification.

### Bleeding Techniques

Bats can be bled by heart puncture or preferably, by the orbital sinus technique previously described on page 21. Wear a glove on the hand used to handle the bat. Generally, 0.2 ml of blood is taken from each bat. Larger amounts of blood may be obtained by holding the bat between the thumb and the first and second fingers and applying pressure to thoracic area. This apparently raises the blood pressure and increases the yield of blood from the orbital sinus.

Serum extraction and storage procedures are as already described.

## BIRD SPECIMENS

### General Considerations

During an epidemic of St. Louis encephalitis (SLE), WE or EE, it may be desirable to determine the extent of arbovirus infection in birds since they are the primary vertebrate hosts and serve as the main sources of virus for mosquito infection. Select a site which contains a variety of habitats common to the area and set up a suitable number of mist nets. Blood samples can be taken periodically from all wild birds captured over the entire epidemic period, or emphasis can be placed upon specific bird populations at particular times, depending upon the extent of ecological knowledge already on hand. In an urban SLE outbreak, for example, it may be desired to study chickens, ducks, geese, pigeons and house sparrows more intensively than other species because of their close association with man. Virus isolation and antibody rates ascertained for each of these species will reveal their degree of involvement. If knowledge of the geographical extent of an epizootic in birds is desired, many collecting sites over a wide area must be sampled.

An investigation of arboviruses in an endemic area can be conducted at a more leisurely pace and with more forethought than epidemic studies. The investigator has more time to plan his program. It may be desirable to set up permanent study areas to permit repeated sampling over several years to determine the many ecological factors which affect cyclical arbovirus activity.

### Permit Requirements

Both federal and state scientific collecting permits are required to capture wild birds and should be obtained in advance of commencing field activities. Federal permits can be obtained by writing to the nearest regional office of the U. S. Fish and Wildlife Service, Bureau of Sports Fisheries and Wildlife. Application should be made by the senior investigator who should list the names of individual field workers. A special permit is required to operate Japanese mist nets. After the federal permits have been obtained, application can be made to the appropriate state wildlife agency. Accurate records must be kept since both state and federal agencies require an annual report listing the birds taken and their disposition.

### Collection Methods

1. Japanese Mist Nets

The Japanese mist net is about 40 feet long and 7 feet high, and made of fine, black nylon which is arranged in four shelves or tiers (**Figure 43**). Different mesh sizes are available depending upon the size of the birds to be captured; the size designated as "Type A" is the most useful for arbovirus studies. Sources of Japanese mist nets are listed on page 64.

The mist net operates on the principle of a trammel net, that is, the bird cannot see the fine nylon mesh, flies into the net and is held in a pocket created by excess net material. The more it struggles to escape, the more entangled it becomes.

Strategic placement of nets requires some knowledge of bird habits and ecology. If the capture of many different bird species is desirable, the nets must be placed in a variety of habitats. Some suggestions for net placement are given in the map in **Figure 44.** This map portrays a good collecting area since at least eight types of habitats are present, all within easy walking distance. Scattered net sites which require much driving or hiking to reach should be avoided since the nets may be unattended too long between inspections.

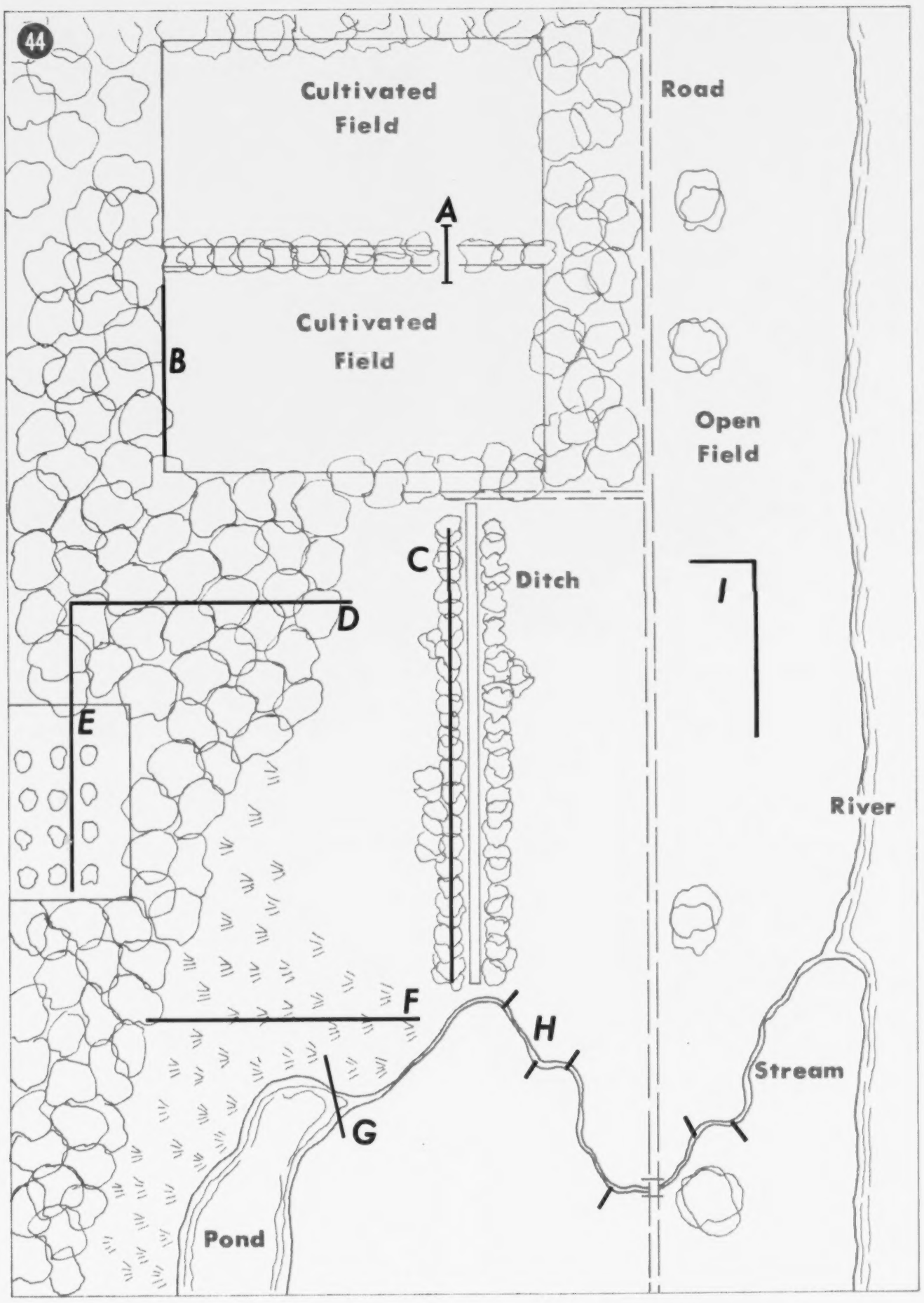
Net site (A) was created by cutting through a hedge row and placing the net at right angles to the row to capture the birds flying along the hedge row. Net arrangement (B) was obtained by placing several nets parallel to the edge of a forested area adjacent to an open or cultivated field. A line of nets was placed in a row of trees along an open ditch to capture tree-top dwelling birds when they come to drink each morning (C). A line of nets in a forest (D) or nets in an orchard (E) will capture different kinds of birds than a net in a swamp (F). Pond (G) and stream habitats (H) offer the attraction of water and an increased supply of insects as food. Visual observation of birds frequently using a particular fly way (H) or actively working over an open field feeding on insects (I) may indicate the desirability of placing nets at these locations. Since there is no cover to conceal the nets in the open, placing at least one net at right angles to the line (I) may increase the catch.

An area approximately 8 feet wide and 40 feet long must be cleared for each net site **(Figure 45)**. A sharp machete is the best tool for this operation. Low vegetation which will become entangled in the lower shelf of the net should be removed. Net poles are inserted into the ground at the approximate expanse of the net **(Figure 46)**. Convenient reusable net poles can be made from 10-foot lengths of ½ inch electrical conduit (EMT) pipe. For transporting convenience, these 10-foot lengths are cut in half and a set-screw coupling fastened to the bottom half. The loops on one end of the mist net are slipped over the disjointed upper half of a pole **(Figure 47)**; this upper half of the pole is then inserted into the coupling on the lower half of the pole.

The folded net is then played out as the worker walks toward the other net pole, taking care to keep the net from falling on the ground where it could become entangled with debris **(Figure 48)**. When the far pole is reached, the net end loops are placed on the lower pole which is set in a position determined by the desired tautness of the net. The loops are then adjusted on the top half of the pole which is placed in position on the coupling. The distance between loops in a properly set net varies according to the net and wind conditions of the day, but generally, the distance should permit an ample pocket for capturing the birds. If the net is too tight, the birds striking the net bounce back out and fail to become entangled.

Several nets may be placed in a line by starting the second net on the second pole of the first net and continuing in this manner, adding as many nets as desired. Generally no more than 30-35 nets can be operated efficiently by a two-man team. Once the nets are opened, visits should be scheduled hourly to remove captured birds before mortality occurs. *Nets should never be left unattended.* Heat from direct sunlight is one of the principal causes of bird deaths in nets. Rain also causes bird mortality. Predators such as cats and raccoons will destroy captured birds and will also tear the nets in the process of removing them. The nets should be furled each evening, and in an area where deer or cattle are prevalent, the nets should be furled as high as possible to keep these large animals from walking through them. The nets should be opened again at daylight, since shortly thereafter bird activity is usually great.

To capture resident species of birds most effectively, the mist nets should be relocated every four to seven days since the birds soon


44
Cultivated Field
Road
A
Cultivated Field
B
Open Field
C
Ditch
I
D
E
River
F
H
Stream
G
Pond

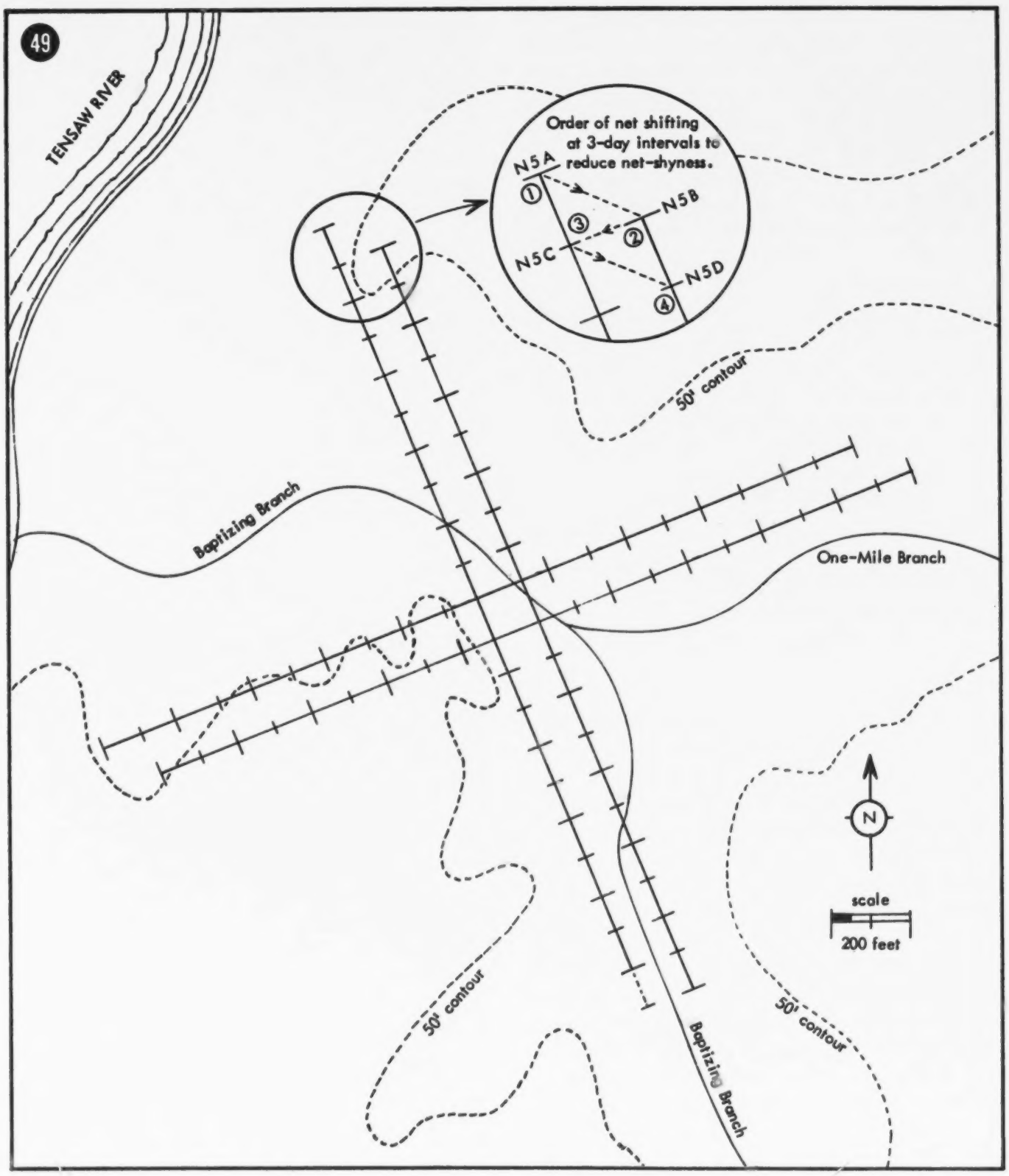
45

47

46

48

learn to avoid the net (**Figure 49**). Some will even continue to avoid the site after the net has been removed. Migrating birds passing through an area probably do not remain in the area long enough to become net shy; therefore, nets for migrants need not be moved except to improve their efficiency. In areas of prevailing winds, adjustments should be made so that the nets are placed across the wind to avoid piling up the loose net at one end.



Various grains or pieces of bread may be used to bait nets for certain species such as blackbirds, sparrows and grackles. Placing three nets in a triangular position with the bait in the center is particularly effective.

The removal of a bird from a mist net is a technique which usually frustrates the beginner. The first step is to determine which side of the net the bird entered, since the bird must be removed from that direction. Then grasp the bird in one hand, holding its head to prevent biting or pecking. With the other hand, free one leg first, then hold this leg clear with a finger of the first hand until the other leg is freed. Next, remove the net from the tail, then from the wings, and lastly from the head.

2. Bait Traps

A manual is available which describes many types of bait traps for capturing birds (4). In general, these traps are made of hardware cloth and are designed so that the birds enter a small opening through which they are unable to escape. The presence of one or more birds in the trap generally enhances its effectiveness. Thus, it may be useful to leave one or two birds as decoys. Some species, however, have distress calls that may frighten away other potential captures.

3. Bal-chatri Trap

The Bal-chatri trap is used in capturing birds of prey (12). This trap is a small wire cage, heavily weighted with lead, containing a live mouse or a small bird as bait. Numerous nylon slip knot nooses are fastened to the wire cage, each opened to snare the toes of birds of prey attempting to grab the small animal inside the cage. Usually the bird gets caught first by one foot, and then the other foot is entangled in the struggle to escape.

4. Cannon Net

The cannon net is used to capture species of birds which can be baited in large numbers such as blackbirds, pigeons, ducks, and vultures. The net is a large type of fish net with a one-inch square mesh, and is operated by placing three cannons in a line with the net folded in front of the cannons (**Figure 50**). The bait is placed in front of the net

(**Figure 51**). The projectiles of the cannons are fastened to the leading edge of the net, while the trailing edge is anchored to the ground. When a sufficient number of birds have been attracted to the bait, the operator located in a blind nearby fires the cannons simultaneously by electrical impulse. The projectiles carry the net up and over the birds

(**Figure 52**) to settle down on them. Usually most of the birds are captured when the cannons are fired properly. If some escape, the projectiles have been fired too vertically; if the birds are hit, the projectiles have been fired too horizontally. Instructions for the use of the cannon nets are contained in *A Field Guide to Cannon Net Trapping* (13). The Appendix contains a list of equipment and suppliers for the cannon net.

5. Shooting

Despite the general effectiveness of nets and traps, some species of birds elude capture. As a last resort, birds may be taken by using a 16- or 12-gauge shotgun loaded with No. 7½-9 shot. In addition to the obvious disadvantage of killing the bird, non-specific serological reactions may occur in blood samples taken from birds that are shot. Results obtained from such samples must be viewed with this in mind.

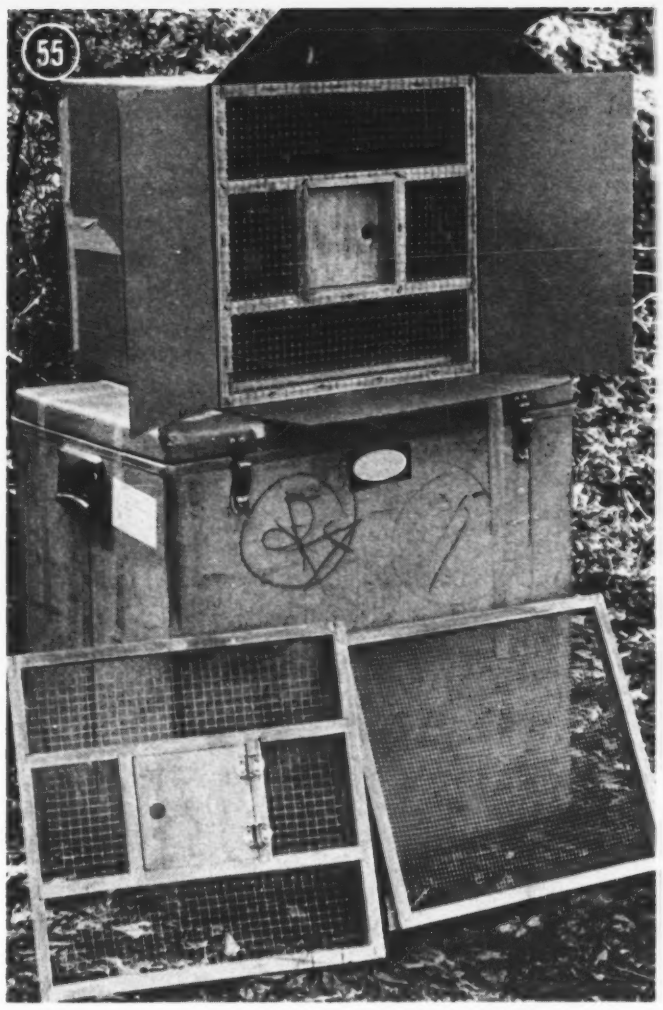
6. Collection from Nests

Nestling birds may be taken directly from the nest. Some species such as house sparrows and finches nest in either buildings or trees; others such as robins and doves nest in trees; still other species nest on the ground, under bridges and in a variety of other habitats. Usually ladders to reach high places and bags for carrying the nestlings down from the nest are the only special equipment needed to collect samples from nestling birds. After the blood samples are obtained the nestlings should be carefully replaced.

**Holding Techniques**

Some species of birds apparently withstand capture and handling without apparent ill effects, but for others, these procedures may be traumatic. In either case, it is strongly recommended that all captured birds be released as soon as banding or marking and bleeding are completed. Frequently, several birds may be captured simultaneously, requiring that some must be held for short periods until they can be processed. Temporary holding bags made of black cotton cloth are useful for this purpose (**Figure 53**). A convenient size is about 16″ square, with either draw strings or a pair of 1″ flexible corset stays sewn in at the mouth of the bag to keep it closed. A number of these bags are required so that no more than three or four birds need be held in one bag. Birds that are retained should be kept in a cool place *at all times.*

A collapsible wire fish basket can also be used, but no more than five or six birds should be held in it at one time (**Figure 54**).




A convenient holding box for birds can be constructed from a 18″ x 18″ x 18″ cardboard carton (**Figure 55**). An insert of ½″ hardware cloth mounted on a 1″ wooden frame is fitted to one side of the box. This serves as the bottom when the box is set on its side. Another frame, similarly covered with hardware cloth but with a spring-loaded trap door, is fit snugly into the top of the box. This frame is held into place by pins or bolts which are slipped into holes drilled through cardboard and frame at suitable points. Newspapers are placed under the bottom frame for rapid cleaning. An advantage of both the black bag and the cardboard box cage is that birds are less active in the dark. Woodpeckers and grosbeaks *should be kept separate* from smaller birds since they may injure them. Obviously, birds of prey should be held separately as well.

**Identification and Age Determination**

A number of books may be consulted for bird identification (14-22). Age determination of birds, although sometimes difficult can add useful information to an arbovirus ecology study. No single method is adequate. Some bird plumages are distinct in the immature and sub-adult stages, e.g., in the orioles, herons, and many of the warblers. Many passerine birds have incomplete skull ossification when immature (23), whereas ossification is complete in adults. The formation of bony material commences at the posterior portion of the skull, and as the bird matures, the bone development extends in a forward progression (**Figure 56**). The skull ossification technique is less accurate with non-passerines. Eye color may also indicate age; for example, the eye of the immature mockingbird is gray, while that of the adult is yellow. A guide for the determination of the age of warblers has been published by Robbins (24).

**1**

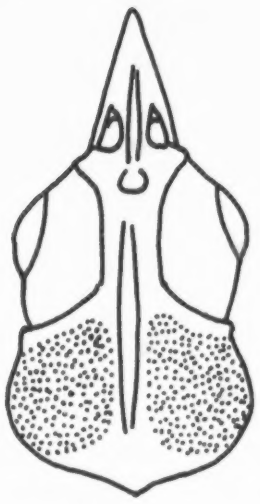
GRASP BIRD GENTLY, BUT FIRMLY IN ONE HAND....

56

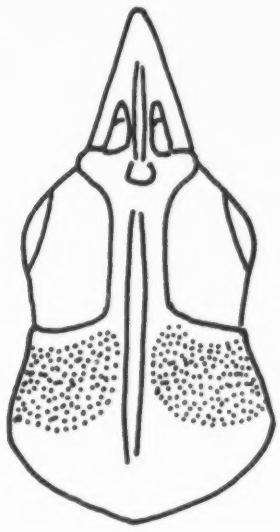
**2**

PULL A FEW FEATHERS AWAY FROM BACK OF HEAD SO THAT LINE OF OSSIFICATION MAY BE SEEN THROUGH SKIN, TO DO THIS, IT MAY BE NECESSARY TO MOVE SKIN BACK AND FORTH OVER SKULL....

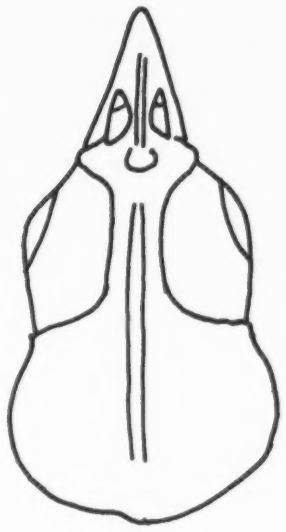
IMMATURE



PARTIALLY OSSIFIED



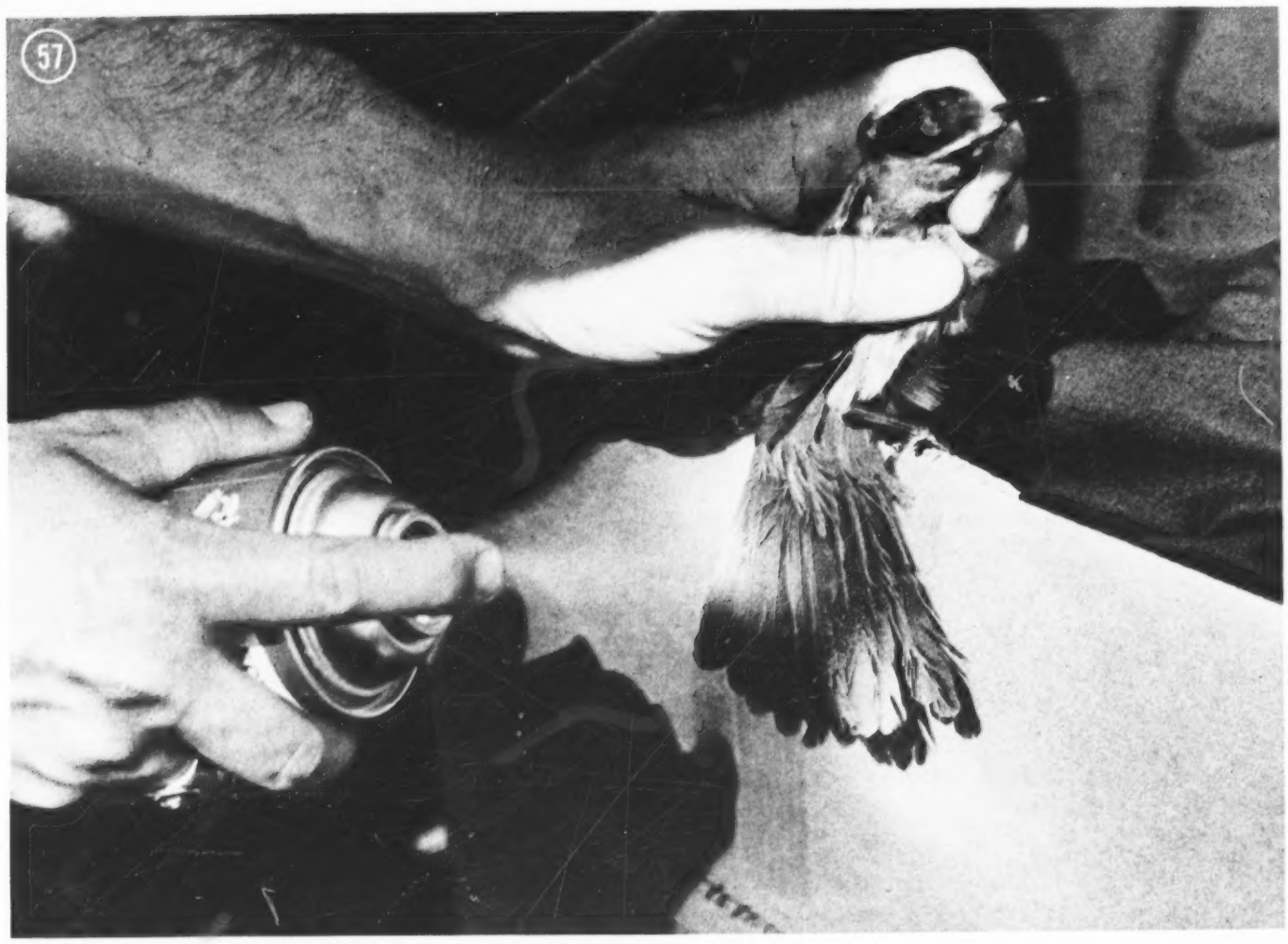
ADULT



**Banding and Marking Techniques**

Recapturing and rebleeding wild birds can furnish useful infection data since a serological conversion during the interval between captures indicates the time the infection took place. A good recapture rate of resident species can be expected if the study is continued over a period of time in the same location. However, very few recaptures of migratory small birds have been made. Considering the effort thus far expended by various workers and the meager results obtained, banding small birds during migration seems unwarranted. However, spraying a portion of the tail of a migratory bird with fluorescent paint aids in recognizing the bird as a recapture if it is taken again (**Figure 57**). This paint dries to a fine powder and lasts about two weeks. Regular enamel spray paint interferes with flight and should *never* be used. Special authorization from the U. S. Fish and Wildlife Service is required to use the fluorescent paint.

Permits are required to band wild birds. These permits are issued by the Banding Office, U. S. Fish and Wildlife Service, Laurel, Maryland 20810. To qualify for a permit, one must be accomplished in bird identification and his competence vouched for by recognized authorities in ornithology. Bands are supplied by the Banding Office, but for certain species such as house sparrows, pigeons and chickens, the Banding Office prefers that their bands not be used. However, the investigator may purchase a supply of bands for this purpose with his own number series and his return address from the National Band and Tag Company, Newport, Kentucky 41071. A list of most North American bird species, with a convenient abbreviation and recommended band sizes for each, is presented in **Figure 58**. The Banding Office requires a detailed report on all banding performed during the year.



58

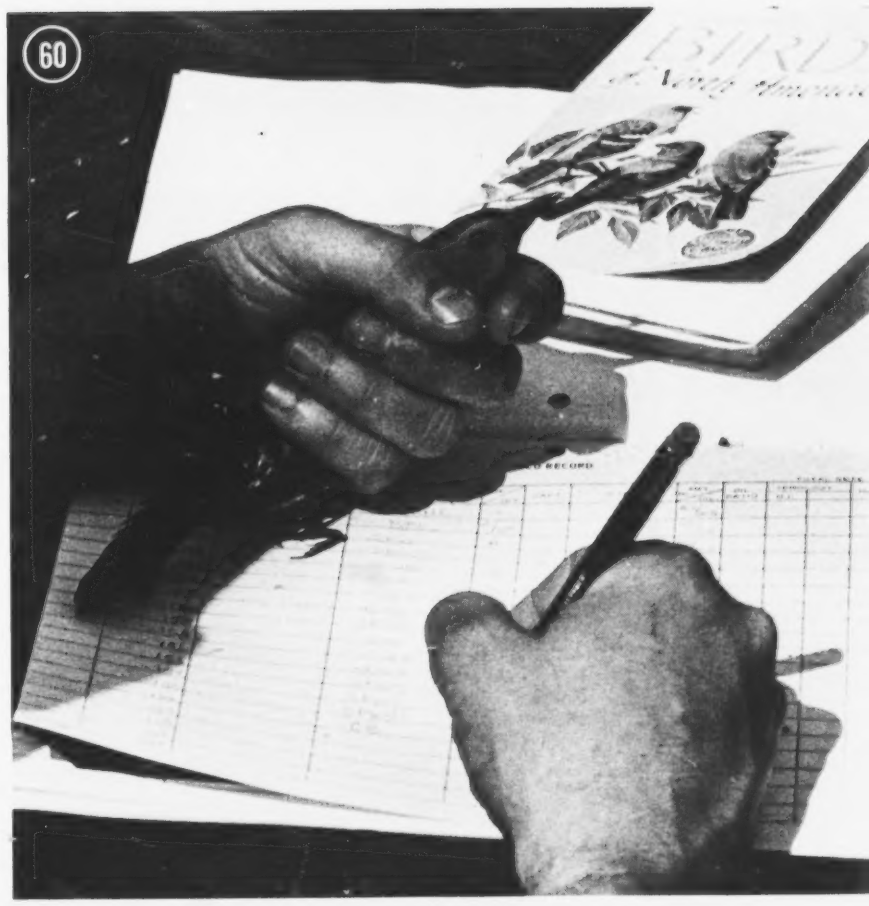
## SPECIES CODE, RECOMMENDED LEG BAND SIZES AND AOU # OF NORTH AMERICAN BIRDS

| | | | |
|---|---|---|---|
| AB | 7B | 081 | ALBATROSS, Black-footed |
| AL | 7B | 082 1 | Layson |
| A | 8 | 118 | ANHINGA |
| AS | 4 | 383 | ANI, Smooth-billed |
| AR | 6 | 032 | AUK, Razorbill |
| AUC | 3 | 016 | AUKLET, Cassin s |
| AA | 4 | 225 | AVOCET, American |
| BIA | 6 | 190 | BITTERN, American |
| BIL | 4 | 191 | Least |
| BBB | 2 | 510 | BLACKBIRD, Brewer's |
| BBRW | 2 | 498 | Redwinged |
| BBR | 2 | 509 | Rusty |
| BBT | 2 | 500 | Tricolored |
| BBY | 2 | 497 | Yellow-headed |
| BLE | 1B | 766 | BLUEBIRD, Eastern |
| BLM | 1B | 768 | Mountain |
| BLW | 1B | 767 | Western |
| B | 1B 1A | 494 | BOBOLINK |
| BW | 3A | 289 | BOBWHITE |
| BOBF | 7B 8 | 114 | BOOBY, Blue faced |
| BOB | 7A | 115 | Brown |
| BOR | 7B | 116 | Red Footed |
| BR | 7B | 173 | BRANT |
| BRB | 7B | 174 | Black |
| BH | 6 | 153 | BUFFLEHEAD |
| BTI | 1 | 598 | BUNTING, Indigo |
| BTLA | 1A | 605 | Lark |
| BTL | 1 | 599 | Lazuli |
| BTP | 0 | 601 | Painted |
| BTS | 1B | 534 | Snow |
| BC | 0 | 743 | BUSHTIT, Common |
| C | 7A | 147 | CANVASBACK |
| CA | 1A | 593 | CARDINAL |
| CB | 1A | 704 | CATBIRD |
| CC | 5 | 311 | CHACHALACA |
| CY | 1B | 683 | CHAT, Yellow breasted |
| CHBC | 0 | 735 | CHICADEE, Black capped |
| CHB | 0 | 740 | Boreal |
| CHC | 0 | 736 | Carolina |
| CHCB | 0 | 741 | Chestnut backed |
| CHM | 0 | 738 | Mountain |
| CWW | 3 | 416 | CHUCK WILL S WIDOW |
| COOT | 6 | 221 | COOT, American |
| COB | 8 | 122 | CORMORANT, Brandt s |
| COD | 8 | 120 | Double crested |
| COG | 8 | 119 | Great |
| CBR | 1A | 596 | COWBIRD, Bronzed |
| CBH | 1A | 495 | Brown headed |
| CS | 9 | 205 | CRANE, Sandhill |
| CRB | 0 | 726 | CREEPER Brown |
| CRBR | 1B | 521 | CROSSBILL Red |
| CRBW | 1 | 522 | White winged |
| CRC | 5 | 488 | CROW, Common |
| CRF | 5 | 490 | Fish |
| CUB | 2 | 388 | CUCKOO, Black billed |
| CUM | 3 | 386a | Mangrove |
| CUY | 2 | 387 | Yellow billed |
| CLB | 5 | 268 | CURLEW Bristle thighed |
| CLL | 4 | 264 | Long billed |
| DC | 1 | 604 | DICKCISSEL |
| D | 1A | 701 | DIPPER |
| DOG | 2 | 320 | DOVE Ground |
| DOI | 2 | 321 | Inca |
| DOM | 3A | 316 | Mourning |
| DORT | 3 | 315 2 | Ringed turtle |
| DOR | | 313 1 | Rock |
| DOS | 4 | 315 | Spotted |
| DOWF | 3A | 318 | White fronted |
| DOWW | 3A | 319 | White winged |
| DK | 2 | 034 | DOVEKIE |
| DWL | 2 | 232 | DOWITCHER Long billed |
| DWS | 2 | 231 | Short billed |
| DUB | 7A | 133a | DUCK Black |
| DUBH | 7B | 133 | Black Hand reared |
| DUM | 7A | 133 1 | Mexican |
| DUMO | 7A | 134 | Mottled |
| DURN | 6 | 150 | Ring necked |
| DUR | 6 | 167 | Ruddy |
| DUW | 5 | 144 | Wood |
| DL | 1A | 243 | DUNLIN |
| EB | 9 | 352 | EAGLE Bald |
| EG | 9 | 349 | Golden |
| EGCA | 6 | 200 1 | EGRET Cattle |
| EGC | 7B | 196 | Common |
| EGS | 6 | 197 | Snowy |
| EIC | 7B | 159 | EIDER Common |
| FP | 7A | 356 | FALCON Peregrine |
| FPR | 6 | 355 | Prairie |
| FIB | 1B | 526 | FINCH Brown capped Rosy |
| FIC | 1 | 518 | Cassin s |
| FIG | 1B | 524 | Gray crowned Rosy |
| FIH | 1 | 519 | House |
| FIP | 1 | 517 | Purple |
| FLG | 3 | 414 | FLICKER Gilded |
| FLR | 3 | 413 | Red shafted |
| FLY | 2 3 | 412a | Yellow shafted |
| FCA | 0 | 465 | FLYCATCHER, Acadian |
| FCAT | 1A | 454 | Ash throated |
| FCB | 0 | 472 | Beardless |
| FCD | 0 | 469 | Dusky |
| FCG | 0 | 469 1 | Gray |
| FCGC | 1B | 452 | Great Crested |
| FCH | 0 | 468 | Hammond s |
| FCL | 0 | 467 | Least |
| FCO | 0 | 455a | Olivaceous |
| FCOS | 1 | 459 | Olive-sided |
| FCS | 1A | 443 | Scissor-tailed |
| FCT | 0 | 466 | Traills |
| FCU | 0 | 467 9 | Unident Empidonax |
| FCV | 1 | 471 | Vermilion |
| FCW | 0 | 464 | Western |
| FCWC | 1A | 453 | Wied's Crested |
| FCY | 0 | 463 | Yellow bellied |
| FBM | 7A | 128 | FRIGATE-BIRD, Magnificient |
| F | 6 | 086 | FULMAR |
| G | 6 | 135 | GADWALL |
| GAC | 6 | 219 | GALLINULE, Common |
| GAP | 6 | 218 | Purple |
| GT | 8 | 117 | GANNET |
| GCT | 0 | 752 | GNATCATCHER, Black-tailed |
| GCB | 0 | 751 | Blue-gray |
| GWB | 3A 4 | 250 | GODWIT, Bar-tailed |
| GWM | 4 | 249 | Marbled |
| GEB | 6 | 152 | GOLDENEYE, Barrow s |
| GEC | 6 | 151 | Common |
| GEU | 6 | 151 2 | Unidentified |
| GFA | 0 | 529 | GOLDFINCH, American |
| GFLA | 0 | 531 | Lawrence s |
| GFL | 0 | 530 | Lesser |
| GOB | 7B | 169 1 | GOOSE, Blue |
| GOC | 8 | 172 | Canada |
| GOE | 7B | 176 | Emperor |
| GOR | 7A | 170 | Ross |
| GOS | 7B | 169 | Snow |
| GOW | 8 | 171 | White-fronted |
| GH | 7B | 334 | GOSHAWK |
| GRB | 4M3F | 513 | GRACKLE, Boat-tailed |
| GRC | 3 | 511 | Common |
| GRE | 6 | 004 | GREBE, Eared |
| GRH | 6 | 003 | Horned |
| GRP | 6 | 006 | Pied-billed |
| GRR | 7A | 002 | Red-necked |
| GRW | 7B | 001 | Western |
| GBBH | 1A | 596 | GROSBEAK, Black headed |
| GBB | 1B | 597 | Blue |
| GBE | 1A | 514 | Evening |
| GBP | 2 | 515 | Pine |
| GBR | 1A | 595 | Rose breasted |
| GSB | 5 | 297 | GROUSE, Blue |
| GSR | 6 | 300 | Ruffed |
| GSS | 7A | 309 | Sage |
| GSST | 6 | 308 | Sharp tailed |
| GMB | 4 | 027 | GUILLEMOT, Black |
| GMP | 4 | 029 | Pigeon |
| GUB | 4 | 060 | GULL, Bonaparte s |
| GUC | 5 | 053 | California |
| GUF | 4 | 059 | Franklin s |
| GUG | 7B | 042 | Glaucous |
| GUGW | 7B | 044 | Glaucous-winged |
| GUGB | 7B | 047 | Great Black backed |
| GUH | 6 | 051 | Herring |
| GUL | 5 | 058 | Laughing |
| GURB | 5 | 054 | Ring-billed |
| GUS | 3 | 062 | Sabine s |
| GUU | 5 | 053 4 | Unidentified |
| GUW | 6 | 049 | Western |
| HAB | 5 | 343 | HAWK, Broad winged |
| HAC | 5 | 333 | Cooper s |
| HAF | 7B | 348 | Ferruginous |
| HAH | 7A | 335 | Harris |
| HAM | 5 | 331 | Marsh |
| HAP | 4 | 357 | Pigeon |
| HARS | 6 | 339 | Red shouldered |
| HAR | 7B | 337 | Red tailed |
| HARL | 7B | 347 | Rough legged |
| HASS | 2M3F | 332 | Sharp shinned |
| HAS | 3A 3 | 360 | Sparrow |
| HASW | 7B | 342 | Swainson s |
| HEBC | 7A | 202 | HERON, Blk crowned Night |
| HEGB | 7B | 194 | Great Blue |
| HEG | 4 6 | 201 | Green |
| HELB | 6 | 200 | Little Blue |
| HEL | 6 | 199 | Louisiana |
| HEY | 7A | 203 | Yellow crowned Night |
| HRT | | 428 | HUMMINGBIRD Ruby throated |
| IG | 6 | 186 | IBIS Glossy |
| IW | 6 | 184 | White |
| IWF | 6 | 187 | White faced |
| JAL | 4 | 038 | JAEGER, Long tailed |
| JAP | 3A | 037 | Parasitic |
| JB | 3 | 477 | JAY Blue |
| JF | 2 3 | 479 | Florida scrub |
| JG | 3 | 484 | Gray |
| JGR | 2 | 483 | Green |
| JM | 3 | 482 | Mexican |
| JP | 3 | 492 | Pinon |
| JS | 2 3 | 481 | Scrub |
| JST | 3 | 478 | Steller s |
| JUG | 1 | 570b | JUNCO Gray headed |
| JUM | 1 | 570 | Mexican |
| JUO | 0 | 567a | Oregon |
| JUS | 0 | 567 | Slate colored |
| JUW | 1 | 566 | White winged |
| K | 1A | 273 | KILLDEER |
| KBE | 1B | 444 | KINGBIRD, Eastern |
| KBW | 1A | 447 | Western |
| KFB | 3A | 390 | KINGFISHER, Belted |
| KLG | 0 | 748 | KINGLET, Golden Crowned |
| KLR | 0 | 749 | Ruby-crowned |
| KM | 5 | 329 | KITE, Mississippi |
| KW | 5 | 328 | White-tailed |
| KWB | 4 | 040 | KITTIWAKE, Black-legged |
| L | 1A | 474 | LARK, Horned |
| LSC | 1 | 538 | LONGSPUR, Chestnut-collared |
| LSL | 1 | 536 | Lapland |
| LSM | 1 | 539 | McCown's |
| LSS | 1 | 537 | Smith's |
| LA | 7B | 010 | LOON, Arctic |
| LC | 8 | 007 | Common |
| LR | 8 | 011 | Red-throated |
| MPB | 4 | 475 | MAGPIE, Black-billed |
| MPY | 4 | 476 | Yellow-billed |
| MA | 7A | 132 | MALLARD |
| MAH | 7B | 132 | Hand reared |
| MP | 1A | 611 | MARTIN, Purple |
| MLE | 3 | 501 | MEADOWLARK, Eastern |
| MLW | 3 | 501 1 | Western |
| MGC | 7A | 129 | MERGANSER, Common |
| MGH | 5 | 131 | Hooded |
| MGR | 6 | 130 | Red-breasted |
| MB | 2 | 703 | MOCKINGBIRD |
| MUC | 6 | 030 | MURRE, Common |
| MUT | 6 | 031 | Thick-billed |
| NKC | 2 | 420 | NIGHTHAWK, Common |
| NKL | 2 | 421 | Lesser |
| NC | 3 | 491 | NUTCRACKER, Clark s |
| NHB | 1 | 729 | NUTHATCH, Brown headed |
| NHP | 1 | 730 | Pigmy |
| NHR | 1 | 728 | Red-breasted |
| NHW | 1B | 727 | White-breasted |
| O | 5 | 154 | OLDSQUAW |
| OB | 1A | 507 | ORIOLE, Baltimore |
| OBU | 1B | 508 | Bullock s |
| OH | 1A | 505 | Hooded |
| OO | 1B | 506 | Orchard |
| OS | 1B | 504 | Scott s |
| OU | 8 | 364 | OSPREY |
| OVB | 1 | 674 | OVENBIRD |
| OWB | 6 | 365 | OWL, Barn |
| OWBA | 7B | 368 | Barred |
| OWBU | 4 | 378 | Burrowing |
| OWE | 2 | 381 | Elf |
| OWF | 3A | 374 | Flammulated |
| OWGG | 8 | 370 | Great Gray |
| OWGH | 8 | 375 | Great Horned |
| OWL | 6 | 366 | Long-eared |
| OWSW | 3A | 372 | Saw whet |
| OWS | 5 | 373 | Screech |
| OWSE | 6 | 367 | Short eared |
| OWSN | 9 | 376 | Snowy |
| OA | 5 | 286 | OYSTERCATCHER, American |
| PG | 3A | 288 1 | PARTRIDGE, Gray |
| PELB | 9 | 126 | PELICAN, Grown |
| PELW | 9 | 125 | White |
| PETA | 1B | 108 | PETREL, Ashy |
| PETL | 1A | 106 | Leach s |
| P | 1A | 620 | PHAINOPEPLA |
| PRN | 2 | 223 | PHALAROPE, Northern |
| PRR | 1A | 222 | Red |
| PRW | 2 | 224 | Wilson s |
| PHR | 6 | 309 1 | PHEASANT, Ring necked |
| PBB | 1 | 458 | PHOEBE, Black |
| PBE | 1 0 | 456 | Eastern |
| PBS | 1B | 457 | Say s |
| PGB | 5 | 312 | PIGEON Band tailed |
| PGR | 5 | 313 | Red billed |
| PGW | 5 6 | 314 | White crowned |
| PT | 6 | 143 | PINTAIL |
| PPS | 1 | 700 | PIPIT, Sprague s |
| PPW | 1 | 697 | Water |
| PLA | 2 | 272 | PLOVER, American Golden |
| PLB | 2 | 270 | Black bellied |
| PLP | 1A | 277 | Piping |
| PLSP | 1B | 274 | Semipalmated |
| PLS | 1B | 278 | Snowy |
| PLU | 3 | 261 | Upland |
| PLW | 2 | 280 | Wilson s |
| PW | 1A | 418 | POOR WILL |
| PCG | 6 | 305 | PRAIRIE CHICKEN, Greater |
| PTR | 5 | 302 | PTARMIGAN Rock |
| PTW | 5 | 301 | Willow |
| PFC | 6 | 013 | PUFFIN Common |
| PFT | 6 | 012 | Tufted |
| PY | 1A | 594 | PYRRHULOXIA |
| QC | 3A | 294 | QUAIL, California |
| QG | 3A | 295 | Gambel s |
| QM | 3A | 292 | Mountain |
| QS | 3A | 293 | Scaled |
| RAC | 5 | 210 | RAIL, Clapper |
| RAK | 5 | 208 | King |
| RAV | 3 | 212 | Virginia |
| RC | 6 | 486 | RAVEN, Common |
| RH | 7A | 146 | REDHEAD |
| RPC | 0 | 528 | REDPOLL Common |
| RPH | 0 | 527 | Hoary |
| RSA | 0 | 687 | REDSTART American |
| RSP | 0 | 688 | Painted |
| RR | 5 | 385 | ROADRUNNER |
| R | 2 | 761 | ROBIN |

| | | | |
|---|---|---|---|
| S | 1A | 248 | SANDERLING |
| SAB | 1A | 241 | SANDPIPER, Baird's |
| SAL | 0 | 242 | Least |
| SAP | 1A | 239 | Pectoral |
| SASM | 1 | 246 | Semipalmated |
| SAS | 1A | 256 | Solitary |
| SASP | 1B | 263 | Spotted |
| SAST | 2 | 233 | Stilt |
| SAW | 1 | 247 | Western |
| SAWR | 1A | 240 | White-rumped |
| SSW | 1A | 404 | SAPSUCKER, Williamson's |
| SSY | 1B | 402 | Yellow-bellied |
| SCG | 6 | 148 | SCAUP, Greater |
| SCL | 6 | 149 | Lesser |
| SC | 6 | 148 9 | Unidentified |
| SCS | 7A | 166 | SCOTER, Surf |
| SCW | 7A | 165 | White-winged |
| SHS | 7A | 095 | SHEARWATER, Sooty |
| SHW | 5 | 096 1 | Wedge-tailed |
| SHO | 5 | 142 | SHOVELER |
| SHL | 2 | 622 | SHRIKE, Loggerhead |
| SHN | 2 | 621 | Northern |
| SIP | 0 | 533 | SISKIN, Pine |
| SKB | 4 | 080 | SKIMMER, Black |
| SKUA | 7A | 035 | SKUA |
| SNC | 3 | 230 | SNIPE, Common |
| SOT | 1A | 754 | SOLITAIRE, Townsend's |
| SORA | 3 | 214 | SORA |
| SPBM | 1 | 575 | SPARROW, Bachman's |
| SPB | 0 | 545 | Baird's |
| SPBC | 0 | 565 | Black-chinned |
| SPBT | 1 | 573 | Black throated |
| SPBR | 0 | 562 | Brewer's |
| SPC | 0 | 560 | Chipping |
| SPCC | 0 | 561 | Clay colored |
| SPD | 1B | 551 | Dusky Seaside |
| SPE | 1B | 688 3 | European Tree |
| SPF | 0 | 563 | Field |
| SPFX | 1A | 585 | Fox |
| SPG | 1B | 554 9 | Gambel's |
| SPGC | 1B | 557 | Golden-crowned |
| SPGH | 0 | 546 | Grasshopper |
| SPHA | 1A | 553 | Harris |
| SPHS | 1 | 547 | Henslow's |
| SPH | 1B | 688 2 | House |
| SPL | 1B | 552 | Lark |
| SPLC | 1 | 548 | Le Conte's |
| SPLN | 1 | 583 | Lincoln's |
| SPN | 1B | 554 8 | Nuttall's |
| SPPS | 1B | 554 7 | Puget Sound |
| SPR | 1 | 580 | Rufous crowned |
| SPSG | 1 | 574 | Sage |
| SPSA | 0 | 542 | Savannah |
| SPSS | 1B | 550 | Seaside |
| SPST | 1 | 549 | Sharp tailed |
| SPSO | 1 1B | 581 | Song |
| SPSW | 1 | 584 | Swamp |
| SPT | 1 | 559 | Tree |
| SPV | 1 | 540 | Vesper |
| SPW | 1B | 554 | White crowned |
| SPWT | 1B | 558 | White-throated |
| ST | 2 | 493 | STARLING |
| SB | 4 | 226 | STILT, Black necked |
| SWBK | 0 | 616 | SWALLOW, Bank |
| SWB | 0 | 613 | Barn |
| SWC | 1 | 612 | Cliff |
| SWR | 0 | 617 | Rough winged |
| SWT | 0 | 614 | Tree |
| SWV | 1 | 615 | Violet-green |
| SWAM | 9 | 178 2 | SWAN, Mute |
| SWAT | 9 | 181 | Trumpeter |
| SWAW | 9 | 180 | Whistling |
| STB | 1A | 422 | SWIFT, Black |
| STC | 1B | 423 | Chimney |
| STV | 1 | 424 | Vaux's |
| STW | 2 | 425 | White-throated |
| TAH | 2 | 609 | TANAGER, Hepatic |
| TASC | 1B | 608 | Scarlet |
| TAS | 1B | 610 | Summer |
| TAW | 1B | 607 | Western |
| TTW | 3 | 259 | TATTLER, Wandering |
| TLB | 5 | 140 | TEAL, Blue-winged |
| TLCI | 5 | 141 | Cinnamon |
| TLCO | 4 | 138 | Common |
| TLG | 4 | 139 | Green-winged |
| TLU | 5 | 140.1 | Unidentified |
| TNA | 1B | 073 | TERN, Aleutian |
| TNAR | 3 | 071 | Arctic |
| TNB | 2 | 077 | Black |
| TNBR | 2 | 076 | Bridled |
| TNC | 5 | 064 | Caspian |
| TNCO | 3 | 070 | Common |
| TNF | 3 | 069 | Forster's |
| TNG | 3A | 063 | Gull-billed |
| TNL | 1A | 074 | Least |
| TNN | 3 | 079 | Noddy |
| TNRS | 3 | 072 | Roseate |
| TNR | 5 | 065 | Royal |
| TNSW | 3A | 067 | Sandwich |
| TNS | 3 | 075 | Sooty |
| THB | 2 | 708 | THRASHER, Bendire's |
| THBR | 2 | 705 | Brown |
| THC | 2 | 710 | California |
| THCR | 2 | 712 | Crissal |
| THCB | 2 | 707 | Curve-billed |
| THL | 2 | 711 | Le Conte's |
| THS | 1A | 702 | Sage |
| TRG | 1B | 757 | THRUSH, Gray-cheeked |
| TRH | 1B | 759 | Hermit |
| TRS | 1B | 758 | Swainson's |
| TRV | 2 | 763 | Varied |
| TRW | 1A | 755 | Wood |
| TTB | 0 | 734 | TITMOUSE, Bridled |
| TTP | 1 | 733 | Plain |
| TT | 1B | 731 | Tufted |
| TOA | 2 | 592 | TOWHEE, Albert's |
| TOB | 2 | 591 | Brown |
| TOG | 2 | 592.1 | Green-tailed |
| TOR | 2 | 587 | Rufous-sided |
| TDB | 6 | 177 | TREE DUCK, Blk-bellied |
| TDF | 7A | 178 | Fulvous |
| TBR | 5 | 113.1 | TROPIC-BIRD, Red-tailed |
| T | 9 | 310 | TURKEY |
| TSB | 2 | 284 | TURNSTONE, Black |
| TSR | 2 | 283 | Ruddy |
| V | 1B | 756 | VEERY |
| VD | 0 | 746 | VERDIN |
| VRB | 1 | 633 | VIREO, Bell's |
| VRBW | 1 | 623 | Black-whiskered |
| VRH | 0 | 632 | Hutton's |
| VRP | 0 | 626 | Philadelphia |
| VRR | 0 | 624 | Red-eyed |
| VRS | 0 | 629 | Solitary |
| VRW | 0 | 627 | Warbling |
| VRWE | 0 | 631 | White-eyed |
| VRY | 0 | 628 | Yellow-throated |
| VLB | 8 | 326 | VULTURE, Black |
| VLK | 9 | 326 1 | King |
| VLT | 8 | 325 | Turkey |
| WY | 0 | 696 | WAGTAIL, Yellow |
| WAA | 0 | 747 | WARBLER, Arctic |
| WAAD | 0 | 656 | Audubon's |
| WABA | 0 | 640 | Bachman's |
| WABB | 0 | 660 | Bay-breasted |
| WABW | 0 | 636 | Black-and-white |
| WAB | 0 | 662 | Blackburnian |
| WABP | 0 | 661 | Blackpoll |
| WABT | 0 | 654 | Black-throated Blue |
| WABY | 0 | 665 | Black-throated Gray |
| WABN | 0 | 667 | Black-throated Green |
| WABL | 0 | 641 | Blue-winged |
| WAC | 0 | 686 | Canada |
| WACM | 0 | 650 | Cape May |
| WACE | 0 | 658 | Cerulean |
| WACS | 0 | 659 | Chestnut-sided |
| WACO | 0 | 647.1 | Colima |
| WACC | 1 | 678 | Connecticut |
| WAGC | 0 | 666 | Golden-cheeked |
| WAGW | 0 | 642 | Golden-winged |
| WAG | 0 | 664 | Grace's |
| WAHE | 0 | 669 | Hermit |
| WAH | 0 | 684 | Hooded |
| WAK | 1 | 677 | Kentucky |
| WAKI | 1 | 670 | Kirtland's |
| WAL | 0 | 643 | Lucy's |
| WAMC | 1 | 680 | Macgillivray's |
| WAMA | 0 | 657 | Magnolia |
| WAMO | 1 | 679 | Mourning |
| WAM | 0 | 655 | Myrtle |
| WAN | 0 | 645 | Nashville |
| WAO | 0 | 651 | Olive |
| WAOB | 0 | 649 | Olive-backed |
| WAOC | 0 | 646 | Orange-crowned |
| WAPM | 0 | 672 | Palm |
| WAPA | 0 | 648 | Parula |
| WAP | 0 | 671 | Pine |
| WAPE | 0 | 673 | Prairie |
| WAPR | 1 | 637 | Prothonotary |
| WAR | 0 | 690 | Red-faced |
| WAS | 1 | 638 | Swainson's |
| WAT | 0 | 647 | Tennessee |
| WATO | 0 | 668 | Townsend's |
| WAV | 0 | 644 | Virginia's |
| WAW | 0 | 685 | Wilson's |
| WAWE | 1 | 639 | Worm-eating |
| WAY | 0 | 652 | Yellow |
| WAYT | 0 | 663 | Yellow-throated |
| WTL | 1 | 676 | WATERTHRUSH, Louisiana |
| WTN | 1 | 675 | Northern |
| WXB | 1A | 618 | WAXWING, Bohemian |
| WXC | 1B | 619 | Cedar |
| WH | 4 | 265 | WHIMBREL |
| W | 1A-3 | 417 | WHIP-POOR-WILL |
| WIA | 6 | 136 | WIDGEON, American |
| WIE | 6 | 137 | European |
| WIL | 4 | 258 | WILLET |
| WOPE | 0 | 461 | WOOD PEWEE, Eastern |
| WOPW | 1 | 462 | Western |
| WCA | 3 | 228 | WOODCOCK, American |
| WPA | 3 | 407 | WOODPECKER, Acorn |
| WPD | 1B | 394 | Downy |
| WPG | 2 | 411 | Gila |
| WPH | 2 | 393 | Hairy |
| WPLB | 1B | 396 | Ladder-backed |
| WPL | 3 | 408 | Lewis |
| WPN | 1B | 397 | Nuttall's |
| WPP | 4 | 405 | Pileated |
| WPRB | 2 | 409 | Red-bellied |
| WPRH | 2 | 406 | Red-headed |
| WPWH | 2 | 399 | White-headed |
| WRB | 0 | 719 | WREN, Bewick's |
| WRCA | 1A | 713 | Cactus |
| WRCN | 1 | 717 | Canon |
| WRC | 1 | 718 | Carolina |
| WRH | 0 | 721 | House |
| WRL | 0 | 725 | Long-billed Marsh |
| WRR | 1 | 715 | Rock |
| WRS | 0 | 724 | Short-billed Marsh |
| WRW | 0 | 722 | Winter |
| WT | 1 | 742 | WRENTIT |
| YG | 3A | 254 | YELLOWLEGS, Greater |
| YL | 2 | 255 | Lesser |
| YT | 0 | 681 | YELLOWTHROAT |

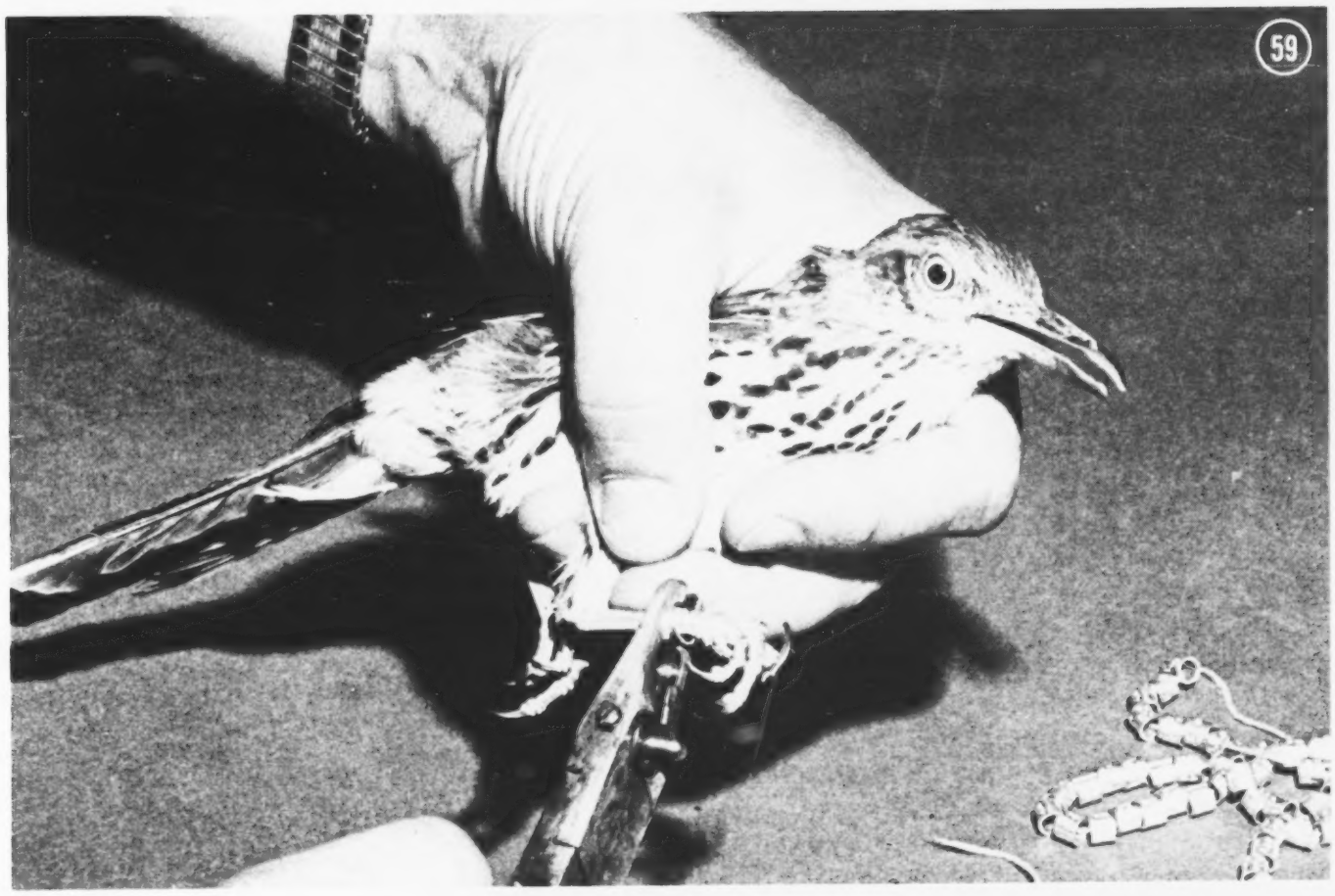
To band a bird, select a band of the correct size, place it upon the leg of the bird, and close it evenly with banding forceps (**Figure 59**). If done carefully, the band will not interfere with the bird's normal activity. On a properly banded bird, the band can be rotated freely on its leg.

Conspicuous markers such as colored plastic back tags and leg streamers can be used to mark birds temporarily. Special authorization from wildlife authorities is required for marking birds.




**Bleeding Techniques**

Pre-dispensing standard amounts of diluent and labeling the tubes prior to bleeding avoids confusion and saves time. Before each bird is bled, all pertinent data are recorded on the data sheet (**Figure 60**). Birds are most frequently bled from the jugular vein, the only large vein in the neck. Hold the bird in the left hand. Gentle blowing on the neck area opens a natural division of feathers and will reveal the jugular vein on the right side. The



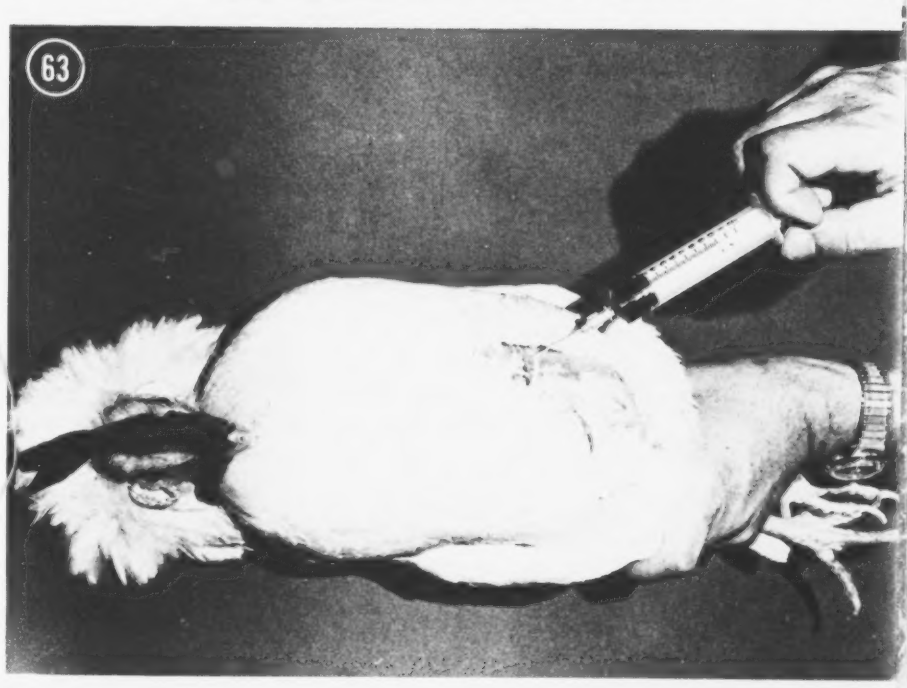
feathers are held apart by the index finger and thumb (**Figure 61**). Holding the bird properly is important in mastering this bleeding technique.

It is not necessary to swab the area with alcohol prior to bleeding. A disposable 25 gauge needle 3/8″ or 5/8″ long is recommended for jugular vein bleeding. Either a 1 ml or 2½ ml disposable syringe is used, depending upon the amount of blood to be drawn. The needle is inserted into the exposed vein, bevel upwards, and slight negative pressure applied. Too much negative pressure will cause the vein to collapse. After the needle is withdrawn, hold some of the downy feathers momentarily over the needle puncture to enhance clotting. When the flow of blood has stopped, *immediately* release the bird.

From birds the size of sparrows and warblers, 0.2 ml of blood is taken and added to 0.8 ml of field diluent. From birds the size of common grackles, 0.5 ml of blood is taken and added to 0.5 ml of field diluent. Chickens and other large birds are bled in amounts of 2.0 to 5.0 ml and the serum obtained undiluted.

Chickens, pigeons, and other large birds can be bled from wing veins (**Figure 62**). Usually one person holds the bird in a convenient position while the second worker draws the blood.

Chickens can also be bled from the heart using a 20 or 22 gauge, 1½″ needle and a 10 ml syringe. One person can conveniently bleed a chicken by holding its feet and wing tips with one hand, while manipulating the syringe with the other (**Figure 63**).



**Sera Processing and Storage**

Frequently, both virus isolation and antibody tests are performed on the bird sera collected; therefore, the blood samples must be handled in such a manner as to preserve any virus present. As described in preceding sections, the blood sample is labeled, allowed to clot for 30 minutes at ambient temperature, then kept cool by water ice or refreezant cans until centrifuged at 2000 rpm for 10-20 minutes. The serum or serum-diluent mixture is poured into another tube which is stored on dry ice in plastic bags or sealed tin cans until delivered to the laboratory. In the laboratory, the serum samples are stored in a mechanical freezer at —70°C until tested for virus. At the completion of these tests, the samples can be transferred to a —20°F freezer if desired to await tests for antibodies.

**Data Recording**

The same Field Record Sheet used to record mammal collection data is used for bird data (**Figure 64**). However, the field numbering system may be modified for birds; for example, FEA8-27 designates a blood sample taken in Florida, Everglades, Avian in the year 1968, and it is the 27th bird captured in that study. If mosquitoes or mammals are not collected from the same area, the "A" representing Avian may be dropped from the code: FE8-27.

The U. S. Fish and Wildlife band numbers or other series of numbers are written in the appropriate column. The abbreviated bird names (Figure 58) are entered in the species column.

**Tissue Dissection**

If special studies require that tissues from birds be tested for virus, place the carcasses in plastic bags and ship them on dry ice to the laboratory for dissection and processing.

**Domestic Flocks**

Additional sources of avian blood samples for serological study are domestic chickens, geese, ducks and turkeys, as well as feral pigeons and certain exotic species kept by bird fanciers. In some areas game farms may rear wild species such as pheasants, quail, chuckars and ducks.

Private flocks offer several advantages: they are already present and exposed to vectors in the area under consideration, minimum effort is required to obtain a reasonable number of samples, and the investigator need not maintain the flock. Disadvantages include embarrassment or liability if some of the birds are injured or killed by handling, dependence upon the particular locality and possibility of their removal before the study is completed.

Presence of antibody in single samples indicates past infection; if the birds are of known age, some indication of the time of infection is obtained. However, much more specific information is obtained from testing repeated samples taken at regular intervals. Unless the owner is particularly cooperative, sentinel flocks may need to be established to obtain the repeated samples.

**Sentinel Flocks**

Sentinel bird flocks can serve a dual survey purpose: first, determination of the level of arbovirus activity in the area by serological conversion; and second, attraction of mosquitoes which can be collected and tested for virus.

The use of sentinel flocks allows the investigator better control of the survey. He can choose the location, establish uniformity of shelter type, assure exposure of roosting birds to mosquitoes, and choose the number and breed, age and source of birds, and control their nutrition. With such factors controlled, conversion rates, field infection rates, and vector abundance can be more reliably compared. A suitable financial arrangement can often be made with a farmer to provide daily care for the birds for a specified period of time.

The standard NCDC sentinel chicken shed (25) has given good results in western United States. This shed (**Figure 65**), constructed of plywood, is six feet square and four feet high, with a flat roof. It contains a

## FIELD RECORD

64

LOCATION: Brownsville Texas COLLECTORS: Newhouse + Johnston TOTAL NETS TRAPS: 35 nets

| DATE | FIELD NUMBER | BAND/TAG NUMBER | SPECIES | AGE/SEX | CAPT. | SITE | AMT. BL./DIL. | DIL. RATIO | SEROLOGY H.I. | SEROLOGY | ISOL. | MISC. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4/17/68 | TB8V 20 | CDC 532 | OO | A/M | N | Linn-Stell Banco | .5/.5 | | | | | |
| | TB8V 21 | CDC 1334 | YT | A/F | N | | .2/.8 | | | | | |
| | TB8V 22 | CDC 1335 | YT | A/M | N | | .2/.8 | | | | | |
| | TB8V 23 | CDC 533 | GRB | A/F | N | Charles Place | 1.0/1.0 | | | | | |
| | TB8V 24 | CDC 1336 | WAC | A/M | N | Linn-Stell Banco | .2/.8 | | | | | |
| | TB8V 25 | CDC 534 | FCWC | A/u | N | | .5/.5 | | | | | |
| | TB8V 26 | CDC 1337 | YT | A/M | N | Charles Place | .2/.8 | | | | | |
| | TB8V 27 | CDC 535 | MB | A/u | N | | .5/.5 | | | | | |
| | TB8V 28 | CDC 536 | FCWC | A/u | N | | .5/.5 | | | | | |
| | TB8V 29 | CDC 1338 | VRWE | A/u | N | | .2/.8 | | | | | |
| | TB8V 30 | CDC 537 | SPO | A/u | N | | .2/.8 | | | | | |
| | TB8V 31 | CDC 538 | OB | I/M | N | | .2/.8 | | | | | |
| 4/18/68 | TB8V 32 | CDC 26 | Golden Fronted Woodpecker | A/M | N | | .5/.5 | | | | | |
| | TB8V 33 | CDC 539 | SPO | A/u | N | | .2/.8 | | | | | |
| | TB8V 34 | CDC 540 | OO | A/M | N | | .2/.8 | | | | | |
| | TB8V 35 | CDC 541 | OO | A/M | N | | .2/.8 | | | | | |
| | TB8V 36 | CDC 542 | DOG | A/u | N | | .5/.5 | | | | | |
| | TB8V 37 | CDC 1339 | YT | A/M | N | | .2/.8 | | | | | |
| | TB8V 38 | CDC 1340 | RSA | A/M | N | | .2/.8 | | | | | |
| 4/19/68 | TB8V 39 | CDC 543 | CA | A/F | N | | .2/.8 | | | | | |

PHS 3.398A (NCDC)
REV. 2-68

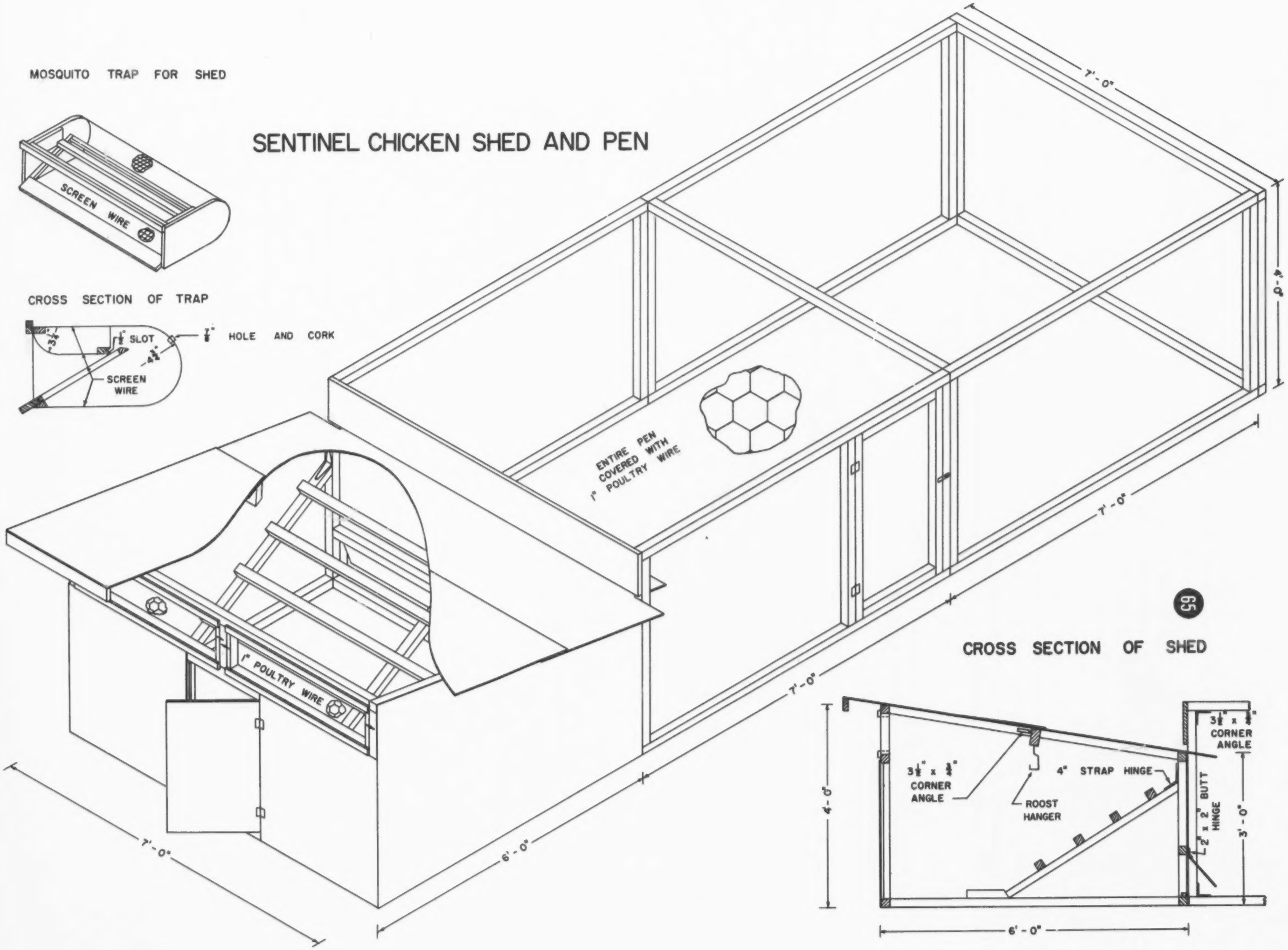
# SENTINEL CHICKEN SHED AND PEN

MOSQUITO TRAP FOR SHED




CROSS SECTION OF TRAP




65

CROSS SECTION OF SHED

roost for the flock and an attached pen four feet high, 14 feet long, and wide enough to provide room for exercise. A ten-inch door at the bottom of the shed permits free movement of the chickens between the roost and pen. Above this door is a large screened window for ventilation.

On the side of the shed opposite the door there is a removable cage with ingress baffle designed to retain mosquitoes attracted from that direction. These can be removed daily for virus tests. Other mosquitoes have free access to the roosting birds through the door and window.

In the spring, chickens six to eight weeks old are pretested and only non-immune birds used as sentinels. Fifteen to 30 chickens may be used in the NCDC sentinel shed.

A prime consideration in formulating plans for sentinel studies is the capability of the laboratory. If the laboratory is incapable of testing the sentinel chicken bloods on a current basis, the temporal advantage is lost and, in fact, the "sentinel" aspect loses its meaning since the term "sentinel" implies advanced warning of virus activity.

Sentinel chickens are fasted for 24 hours prior to bleeding to reduce the fat content in their blood. They are usually bled from the wing vein, taking care to prevent the formation of a hematoma. The skin is disinfected with alcohol and the bleeding done as close to the chicken's body as possible so that if a hematoma forms, some undamaged part of the vein remains farther out on the wing for subsequent bleedings. After taking the sample, blood flow can usually be stopped by pressing several downy feathers over the puncture. Alternate wings are used if the bleeding schedule is frequent. The amount of blood taken varies with the size of the chicken. Approximately 0.5 ml of blood is taken from small chickens and diluted with 2.25 ml of field diluent. After centrifugation this yields nearly 2 ml of a 1:10 dilution of chicken serum. Approximately 5 ml is taken from larger birds which yields about 2.5 ml of undiluted serum. Birds which convert from antibody negative to positive should be replaced by non-immune birds, preferably of the same age.

# REPTILE AND AMPHIBIAN SPECIMENS

**Collecting Methods**

1. Land Turtle

There is no simple way to trap land turtles in large numbers. Turtles are best collected by hand on dirt roads or lightly traveled highways, in woods near stream beds, and near ponds. Good results may be obtained by asking children to collect turtles for a small reward.

2. Water Turtle

Water turtles also may be collected in the habitats listed above. Swimming turtles can be netted with a long-handled fish net, but it is almost impossible to surprise an apparently sleeping turtle as it lies in the sun on a rock or log.

Turtle traps can be constructed by forming a cylinder of strong fish net secured to metal hoops. The trap should be about 30 inches in diameter and four feet long, with a cone-shaped entrance at one end and the other end closed. The cone should protrude into the trap; the turtle swims in through a hole of appropriate size in the apex of the cone. The trap must be staked securely in place just at the surface of the water, and must provide a breathing space above the water for the turtles. Traps fabricated of fish net have the advantage of being more or less collapsible, but if more rigidity is preferred, hardware cloth can be substituted. Traps can also be secured to floats and anchored just at the surface of deeper water. Water turtles are attracted by baiting with aged, raw meat scraps, chicken entrails, or chopped fish. Bait is secured within the trap below the water in a well perforated container.

Another type of trap consists of a drum submerged except for about six to eight inches extending above the water level. A plank is placed over the rim of the drum with one end in the water and the other end at about the center of the drum; the plank must be of such length that it is nearly evenly balanced, with the slightly heavier end in the water; the rim of the drum is the fulcrum.

When a turtle climbs out of the water onto the plank to bask, it passes the fulcrum and is dropped into the drum. The plank then returns to its former position. A float should be provided in the drum for the turtles caught.

3. Lizard

Lizards can be collected in cold weather by searching under rocks, boards, or logs. In warm weather they may be caught by stalking and slapping a hand down over them rapidly or by noosing them. Care must be exercised to prevent severe injury. A lizard noose can be made of strong thread or fine wire at the end of a pole. The noose is lowered in front of the lizard and slowly moved back over its head. A rapid upward snap of the pole catches the lizard by the neck. Lizards should not be caught by the tail, since the tail has many fracture joints.

4. Snake

Snakes may be collected along roads or in their natural habitats under logs, stones, wood or trash piles. Aquatic species are found along margins of streams, marshes, bogs, and ponds. Early in the spring or late in the fall snakes may be found in large numbers around their hibernating sites. To reduce the risk of snake bite, some tool such as a rake, hooked stick, or snake tongs should be used. Poisonous snakes should be captured only by persons experienced in their collection.

A noose for holding a snake securely can be made from a length of metal pipe and some thin steel cable. The cable is attached permanently to the lower end of the pipe and passed through the pipe to form a loop. By pulling on the end of the cable the noose is tightened around the snake's neck.

5. Frog

Frogs are most easily collected at night on margins of ponds and streams. When a flashlight beam is directed into their eyes, they will usually sit quietly enough to be caught by hand or net. This method is most satisfactory the first night; however, uncaught frogs in an area become increasingly wary each time they are disturbed. Bullfrogs can be caught from a boat as they float at the water surface, but usually are quite difficult to catch from the shore. If frogs are abundant, it is possible to trap them in Havahart or National traps partially submerged and facing into the water. Frogs can also be collected along roads following summer showers. Cotton cloth bags about 10"x24" in size are convenient for holding reptiles and amphibians temporarily in the field. Amphibians should be kept moist by wetting the bags or by putting a wet cloth or cellulose sponge in the bag.

**Identification and Marking**

Turtles can be marked by fastening numbered metal tags through the edge of the shell or by painting identification numbers on the shell.

Snakes are marked by notching the scutes or by attaching a size #1 fingerling tag through the jaw. The jaw tag does not tear out and apparently does not interfere with the snake's feeding. Tags can be used similarly to mark lizards and frogs.

**Bleeding Techniques**

Turtles are generally bled by one of two methods, both from the heart. By the first method, they are held with the head pulled to the left; a fairly long needle is then inserted through the membrane at the base of the neck on the right side and directed posteriorly into the heart. With the turtle in this position, the heart lies just above the inner tip of the right coracoid and immediately anterior to the hinge line.

The second method involves drilling a small hole, 1/8" in diameter, through the plastron toward the location of the heart. When a box turtle is placed on its back with the head upward, the heart lies to the left of the midline slightly behind the hinge. Drilling must be done carefully so that the membranes lining the body cavity are not torn. If an electric drill is used, a set-screw stop should be placed near the tip of the bit to prevent penetrating too deeply. Since the hole will allow very little maneuvering of the needle, it should be located with care. This same general technique is also applicable to other types

of turtles. Blood should be drawn slowly into the syringe with a minimum of negative pressure to allow the heart to refill with blood.

Turtle red blood cells are toxic to newborn mice by all inoculation routes and to some tissue culture systems. Mice usually die in less than 24 hours. Recentrifuged serum, even diluted 1:10, often remains toxic enough to cause hair follicle destruction on the head when inoculated intracerebrally into mice.

Snakes also may be bled from the heart. If the snake is restrained and held on its back with at least the anterior half of its body fully extended, the heart can be located by its slow pulsing causing movement of the scutes. It lies about one-fourth the total length of the snake from the head. A snake can be bled by one person by allowing the snake to wrap its tail around a stake and pulling the upper part of the body taut. Snake blood, like that of turtles, may also be toxic to newborn mice and tissue cultures.

Lizards are bled from the heart by inserting the needle behind the pectoral girdle and locating the heart by probing.

Frogs are bled by inserting the needle just behind the pectoral girdle and slightly to the frog's left toward the center of the chest. They withstand loss of a considerable amount of blood. Even when bled until apparently moribund, many apparently recover completely within 24 hours if placed in a container of shallow water.

**Processing, Storing and Shipment**

Aseptic virologic techniques should be used in the processing, storing, and shipping of specimens.

# LABORATORY PROCEDURES

### Facilities for Processing Field-Collected Materials

One of the problems of greatest concern in the processing of field-collected vertebrate specimens in the laboratory is the elimination of faulty techniques or conditions which might cause cross-contamination. To solve this problem at NCDC facilities, a separate laboratory is used exclusively for the processing and storage of the field specimens. No materials known to be virus-infected are permitted in this room. The basic equipment provided, used for no other purpose, includes a refrigerated centrifuge, refrigerator, —70°C mechanical freezer and a biological safety hood.

All virus work on virus strains isolated such as passages and virus identification procedures, is done in separate facilities. Even then, field isolates are not handled concurrently with known infected experimental material.

This same caution applies to rooms in which the newborn mice or tissue cultures are inoculated and kept. Preferably, "primary" inoculations (those of field suspensions) are placed in rooms separate from "passage" inoculations which, of course, are much more likely to be infected with virus. Mice inoculated experimentally with known viruses are not placed in the same room with the field material.

### Formula for Field Diluent

The field diluent generally used by the Arbovirus Ecology Laboratory consists of 25 percent normal rabbit serum in 0.05 M phosphate-buffered physiological saline, pH 7.6 to 7.8, and contains 1.6 mg of streptomycin sulfate and 1,000 units of sodium penicillin G per ml. The high proportion of serum gives this diluent excellent virus stabilizing properties. The formula is given on page 65 of the Appendix.

Ordinarily a supply of diluent is made up in the laboratory each day; however, for field studies it is more convenient to make up a single large quantity (1 to 3 liters), test it for sterility and toxicity, then dispense it in 25 ml amounts into serum bottles and store it at —20°F until needed. It is advantageous to purchase rabbit serum in a large lot from which a sample has been pretested and shown to be free of arbovirus antibodies. Prior to use, the rabbit serum is heat-inactivated at 56°C for 30 minutes. Fetal calf or newborn agamma calf serum are also good viral stabilizers for diluents and are usually free from non-specific inhibitors sometimes found in rabbit serum.

Newborn mice are used extensively in isolating arboviruses; however, the recent trend is increased use of various tissue culture systems. If tissue cultures are employed, all glassware used to make the diluent should be washed according to tissue culture specifications. Before using the diluent, a sample should be tested for sterility and toxicity in newborn mice and several tissue culture systems, as well as in routine bacteriological culture media.

### Specimen Preparation

Two labels are prepared for each sample, one remains on the tube and the other used when the sample is inoculated. Generally no further preparation in the laboratory is required for serum specimens that have been handled properly in the field. If sufficient serum is available for all anticipated tests, the clots are usually discarded; however, if necessary, they can be used for virus isolation tests. They are ground in a small mortar, two to four volumes of diluent added, and the mixture centrifuged. The supernate is then inoculated into newborn mice (reptilian blood is toxic, as mentioned previously).

Frozen organs or tissues are thawed after their arrival at the laboratory and 2-3 gram

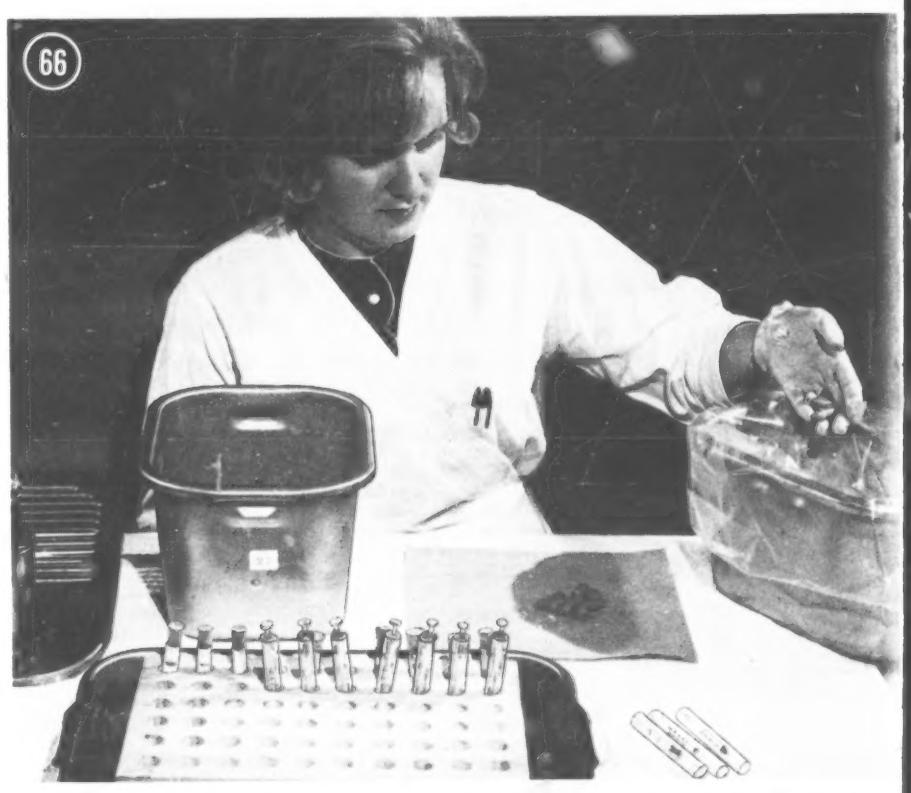
portions removed. A brain sample should preferably include portions of each hemisphere, the cerebellum and the cord. The samples are triturated and a 10% suspension made in the 25% normal rabbit serum diluent containing antibiotics. After centrifugation at 1000 rpm for ten minutes, the supernate is poured off and inoculated into newborn mice or appropriate tissue cultures. The remaining supernate and unground tissue is refrozen and held in reserve until the laboratory work is completed.

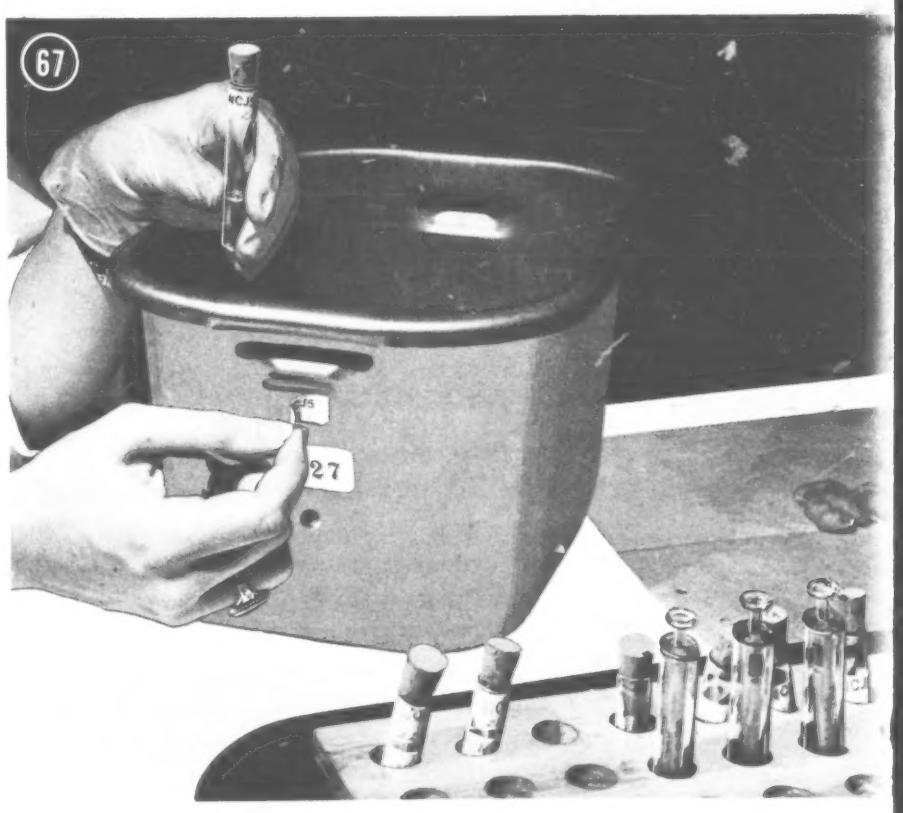
Frozen carcasses of small rodents are removed from their plastic bags and thawed. A small piece of brain tissue weighing approximately 0.2 gm is taken by aseptic techniques as described for mouse dissection. This tissue is ground in 2.0 ml of the diluent, centrifuged and inoculated into newborn mice or tissue cultures. A similar pool may be made up of combined small pieces of heart, liver and spleen.

### Animal Inoculation and Observation

Newborn mice are the system of choice for the isolation of "unknown" arboviruses from vertebrate blood or tissue suspensions because of the lack of a single tissue culture system that equals their broad susceptibility to all kinds of arboviruses. Weanling mice, although satisfactory for some arboviruses, lack the general sensitivity of newborn mice. Pregnant mice are placed in separate cages when they are nearly ready to litter and are checked each morning. The new litters and their mothers are dated and set aside as "zero days old" to be used on day 2 or day 3. They can be inoculated earlier, but the chances of their being destroyed by their mothers is somewhat increased. Mice older than three days are not used to attempt field isolations if the type of virus to be encountered is unknown since resistance develops rapidly with age.

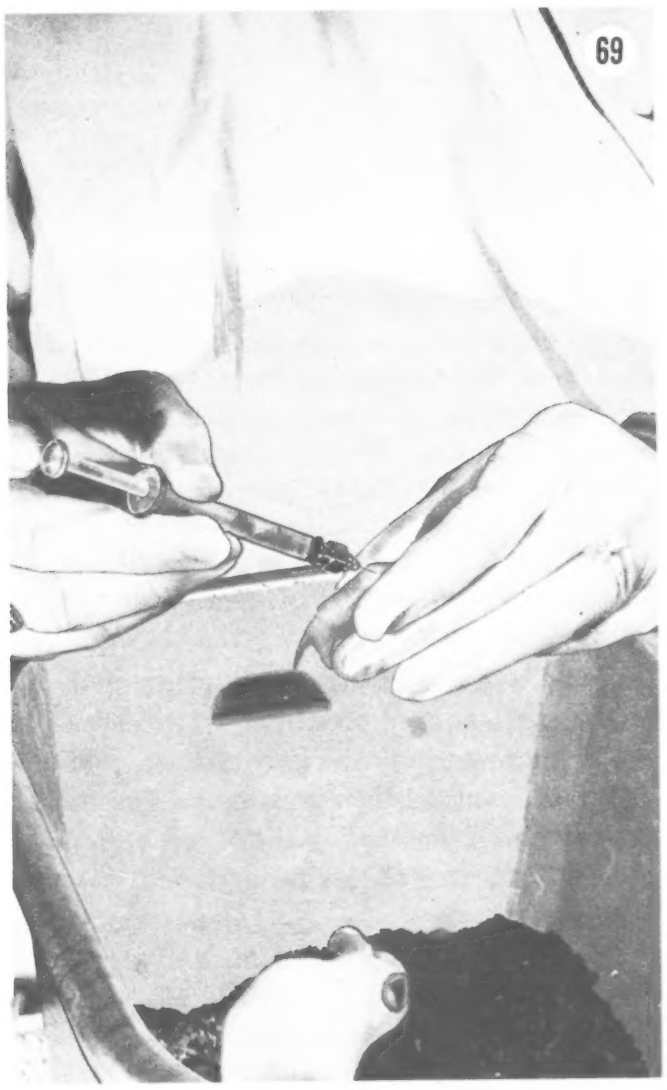
Each litter is culled to six baby mice (**Figure 66**). One of the tube labels is placed on the mouse can (**Figure 67**). The newborn mice are each inoculated intracerebrally with 0.02 ml of the serum sample, using a 1/4 ml tuberculin syringe and 1/4" 27 gauge needle. A separate syringe and needle are used for each blood sample. The syringe is filled (**Figure 68**) and the mice inoculated (**Figure 69**) over the mouse can to minimize contamination of the table area. Special caution is observed to avoid touching the tube lip,

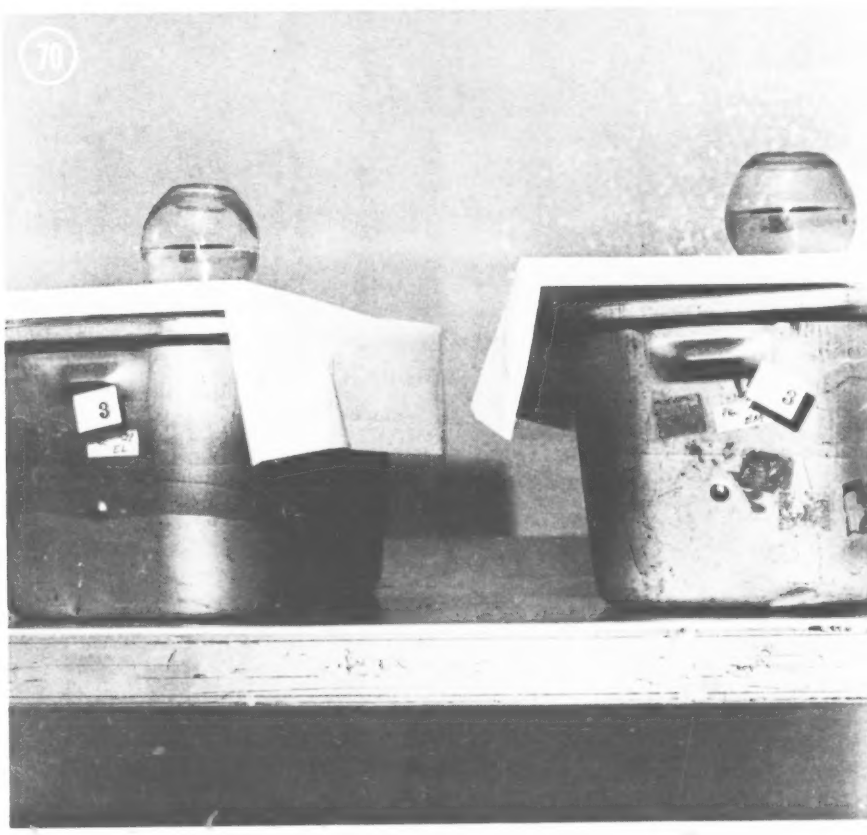


plunger shaft, or needle hub since cross-infections from one litter to the next may occur during inoculation if the hands become contaminated. The hands are braced against the edge of the can for steadiness. A disposable vinyl glove is worn on the hand which holds the mice during inoculation. The tubes of blood samples are kept in a rack in a shallow pan of ice during the animal room operations.

The inoculated mice generally are placed back into the cage with their own mother; however, some technicians pool all the young and place them with mothers at random after inoculation. In fact, the mothers of litters up to a week old may be used by discarding their own young and replacing them with 2- or 3-day-old mice thinned from other litters. Paper filter covers are placed over each cage to reduce the spread of various mouse viruses **(Figure 70)**.





The test mice are checked each morning for 14 days, using wooden tongue depressors **(Figure 71)**. A clean tongue depressor is used for checking each cage. Forceps used to remove dead mice are disinfected before the next use. The mouse count and symptoms are

recorded on an Animal Inoculation Record sheet, using standardized abbreviations: M, missing; E, partially eaten by mother; D, dead; Pa, paralyzed; Pr, prostrate; and Co, convulsing. When symptoms begin to occur, it is wise to check the mice again in the late afternoon. A dash (-) in the checking column indicates that the mice were normal at the time of observation.

A sample Animal Inoculation Record sheet is shown in **Figure 72.** These are kept in loose leaf binders in page order. For convenience, inoculation sheets of field suspensions are kept in a "Primary" book; those of passages are kept in a separate "Passage" book.

The dead mice and those showing signs suggestive of infection are saved for passage or later reference. Usually the mice with severe symptoms, such as general paralysis or prostration, and those which have very recently died, contain more virus in their brains than mice with lesser symptoms. The sick mice are killed by brief exposure to chloroform fumes, then are placed in suitably labeled end-flap 3"x5" manila envelopes (**Figure 73**). The label indicates the mouse involved, symptom, date, and page in the record book. The mice saved are "filed" in date order in a mechanical freezer at —70°C until processed further.

Strict maintenance, sanitation and approved animal care practices are observed in the animal rooms at all times. Ample food and water are constantly available to discourage the mothers from eating their own young. At the close of the observation period, all surviving mice are killed by chloroform. Then they, their bedding material, and any leftover mouse food are incinerated, and the mouse cages and water bottles are sterilized by autoclaving.

72

Date: 5/6/69 Page: 3211

Animal, route, am't inoc.: SM. ic. 0.02 cc

Diluent: 25% NRS + A Exper.: Field Studies Category: Primary

| MATERIAL INOC. | DATE OF CHECKING 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FEV8-27 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| From Bk: page: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| Cultures Br: Susp: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| FEV8-28 | – | – | – | – | – | – | M | | | | | | | | | | | | | | |
| | – | – | – | – | – | – | – | – | M | | | | | | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| From Bk: page: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| Cultures Br: Susp: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| FEV8-29 | M | | | | | | | | | | | | | | | | | | | | |
| | E | | | | | | | | | | | | | | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| From Bk: page: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| Cultures Br: Susp: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| FEV8-30 | – | – | – | – | M | | | | | | | | | | | | | | | | |
| | – | – | – | – | D | saved 5/11/69 3920 Virus + Calif. | | | | | | | | | | | | | | | |
| | – | – | – | – | Pr | M | | | | | | | | | | | | | | | |
| From Bk: page: | – | – | – | – | Pr | D | saved 5/12/69 | | | | | | | | | | | | | | |
| | – | – | – | – | – | Pr/K | saved 5/12/69 | | | | | | | | | | | | | | |
| Cultures Br: Susp: | – | – | – | – | – | Pr/K | | | | | | | | | | | | | | | |
| FEV8-31 | – | D | 3921 | | | | | | Virus + EEE | | | | | | | | | | | | |
| | – | D | saved 5/8/69 | | | | | | | | | | | | | | | | | | |
| | – | D | | | | | | | | | | | | | | | | | | | |
| From Bk: page: | – | D | | | | | | | | | | | | | | | | | | | |
| | – | M | | | | | | | | | | | | | | | | | | | |
| Cultures Br: Susp: | – | M | | | | | | | | | | | | | | | | | | | |
| FEV8-32 | – | – | – | – | – | – | – | – | D | saved 5/15/69 3922 Virus + | | | | | | | | | | | |
| | – | – | – | – | – | – | – | – | – | Pr/K saved 5/16/69 | | | | | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| From Bk: page: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |
| Cultures Br: Susp: | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | |

P. 3219
1st reinoc

Tinsaw

PHS 3 494 (NCDC) ANIMAL INOCULATION RECORD
1-63

**Harvesting Mouse Brains for Passage**

Periodically, usually at weekly intervals, the Animal Inoculation Record sheets are inspected and mice selected from the freezer for brain passage into other suckling mice. A serial code number (passage number) is given each mouse selected and is recorded both on the Animal Inoculation Record sheet and on the mouse envelope. At the same time a passage listing is made out on a Passage List sheet (**Figure 74**), associating the passage number with original suspension, mouse source, and record book pages. Columns also are provided for recording results of sterility tests on brain suspensions. This list is valuable in tracking down the laboratory history of material tested since it shows the source, disposition, dates of handling and association with other materials.

Generally all single, scattered "deads" are passed unless the mice are obviously decomposed or have died of some cause easily recognized as unrelated to arbovirus infection. If more than one mouse in a litter dies in a pattern consistent with virus infection, usually only a single mouse of the group is passed and the remainder held until the outcome is known. If the first mouse passed is negative, a second mouse is passed, provided the symptom or day of death was different from that of the first mouse.

The goal is a distinct positive or negative result from each animal specimen inoculated, but frequently a definite negative test is difficult to obtain. Ideally, a specimen is considered negative if all mice inoculated survive for the full checking period, or if dead mice that are involved are passed with negative results. In many instances, however, nonspecific deaths may have occurred so early in the majority of mice in an inoculated litter that the test is considered unsatisfactory, in which case the original specimen must be reinoculated.

Also, the mother mice may eat some of their young. This is usually not critical if only one or two are eaten on the first day of inoculation; four survivors past the first day generally are considered a legitimate test since few arboviruses kill newborn mice within one day. When missing mice occur at later incubation periods, however, or when an arbovirus is suspected which kills within one day, a strong possibility exists that they were eaten because they were sick. These specimens must be reinoculated.

Some blood specimens appear to be toxic and kill mice on the first day. If this occurs after the second inoculation and passage fails to indicate the presence of a virus, the material should either be diluted 1:10 for reinoculation or placed in hamster kidney or duck embryo tissue cultures. It also may be inoculated into newborn mice by the subcutaneous or intraperitoneal routes, since most arboviruses which kill by the intracerebral route also are infectious when inoculated peripherally. Mice are less subject to toxicity of materials when inoculated peripherally.

The virus positive specimens are recognized more easily since within one or two passages, it will generally kill all mice within a two or three day period of each other. The brains of some of these mice are used for identification by complement fixation, hemagglutination, and neutralization tests. Some also are saved as virus stock for future reference. When a blood sample has been found to contain virus, the original blood sample generally is reinoculated to confirm the validity of the isolation.

PHS 3.491 (CDC)
10-62

74

PASSAGE LIST

Page 301

Date listed: 5/26/69 Date ground: 5/27/69 Date inoc: 5/28/69

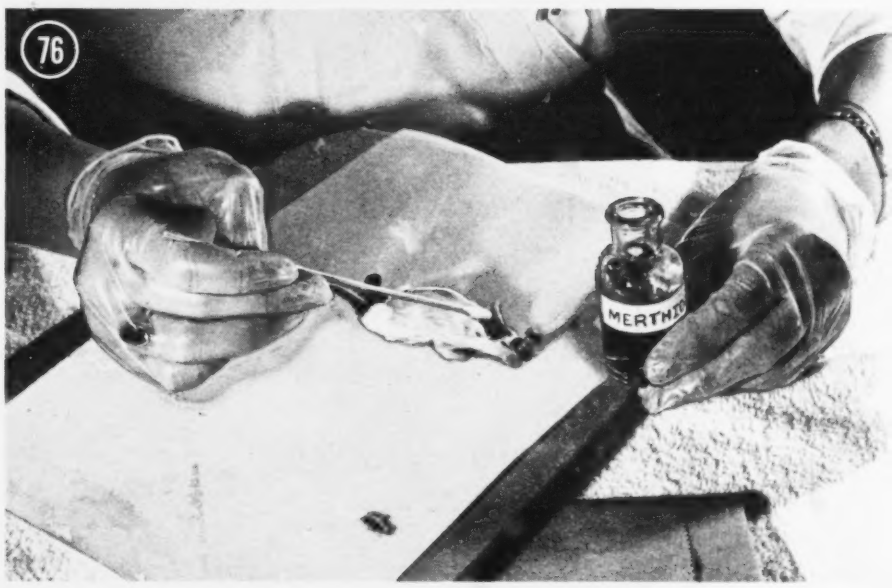
Date dissected: 5/27/69 Host: SM Route: ic Vol: .02 cc

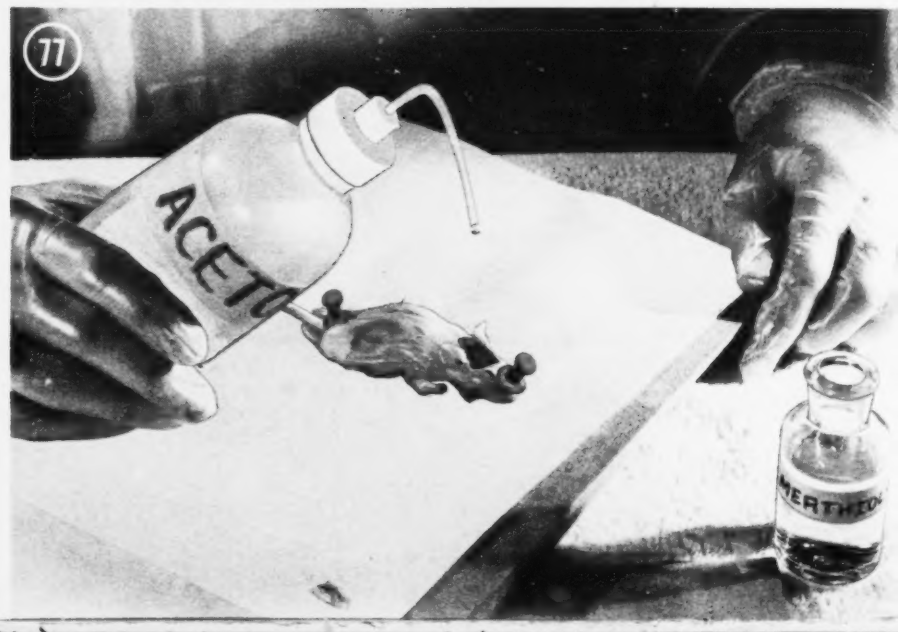
| Date of Death | Laboratory Designation | Cultures Code | Br. | Susp. | Record Books From No. Page | To No. Page | |
|---|---|---|---|---|---|---|---|
| 4/14 | GWV8-39-3SM1-1SM2-4SM3 | 3914 | - | - | Pass 863 | Pass 921 | |
| 4/18 | GWV8-44-5SM1 | 3915 | + | - | Prim 3188 | " | |
| 4/18 | GWV8-76-2SM1 | 3916 | - | - | 3194 | " | |
| 5/1 | TBV8-1302-2SM1 | 3917 | - | - | 3195 | " | |
| 4/28 | TBV8-1491-1SM1 | 3918 | - | - | 3207 | " | |
| 5/2 | TBV8-1501-6SM1-3SM2 | 3919 | + | - | Pass 887 | " | |
| 5/11 | FEV8-30-2SM1 | 3920 | + | - | Prim 3211 | 922 | |
| 5/8 | FEV8-31-1SM1 | 3921 | - | - | 3211 | " | |
| 5/15 | FEV8-32-1SM1 | 3922 | - | - | 3211 | " | |

It is during the passaging procedure that laboratory contamination and serious error are most likely to occur. Infected newborn mouse brain tissue often contains very high concentrations of virus; titers of $10^{9.0}$ with some viruses are not unusual. This means that the material could be diluted a billion-fold and still contain enough virus to kill newborn mice. In handling viruses in such concentration, considerable caution must be taken to prevent risk to the technician and cross-contamination between different brain specimens. Carelessness during the preparations can result in apparent "isolations" from associated non-infected samples, leading to considerable confusion and serious misinterpretations.

A biological safety cabinet with an exhaust system should be used for all procedures. Dissecting instruments should be sterilized by boiling for 20 minutes in an instrument sterilizer. Two sets are used for each mouse, one to remove the skin from the crainium and the other to take out the brain. The method described below can be used for either newborn or weanling mice.

The frozen mice are partially thawed, dipped in 2 percent Lysol solution, and blotted on paper toweling. Then they are pinned to a softwood dissecting board that is covered with a paper towel and a piece of butcher parchment paper. Map pins are used to fasten the mouse down: one is placed through the bridge of the nose and another through the base of the tail. The scalp is removed with the first set of instruments **(Figure 75)**. These instruments are then wiped on a gauze pad and stacked in a jar of 2 percent Lysol disinfectant until sterilized later by boiling. The skull cap and adjacent skin are swabbed with 1:1000 Merthiolate and the swabstick is discarded **(Figure 76)**. A drop or two of acetone is released from a dropper about two inches from the skull to wash away the excess Merthiolate and to cause rapid drying of the skull surface **(Figure 77)**. It is dropped from this height to minimize contamination of the dropper tip, since the same dropper is used for each mouse. A second set of instruments is then used to cut off the skull cap and



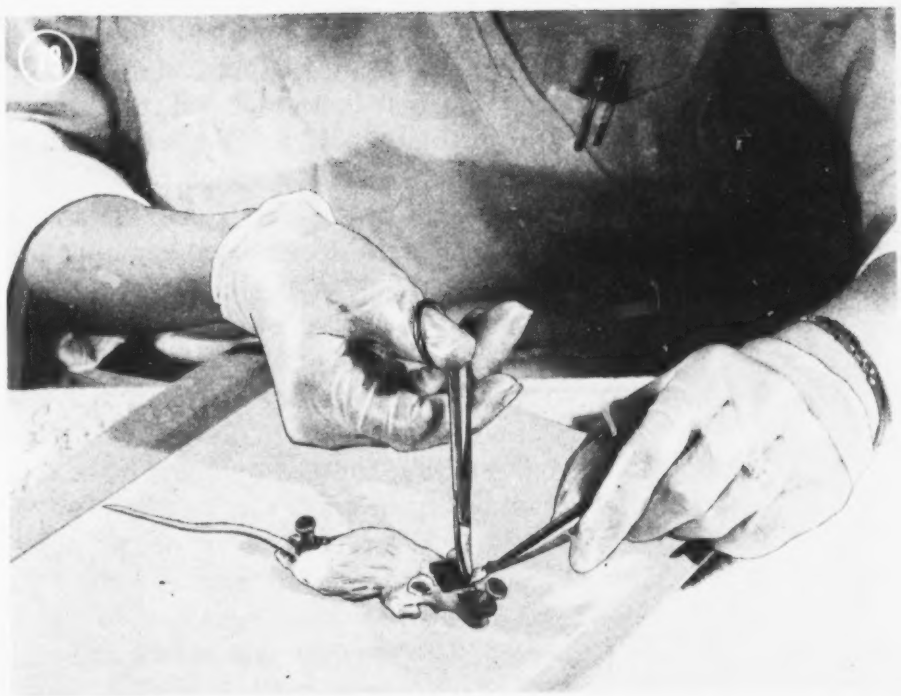
take out the brain (**Figure 78**). It is advisable to have the brain only partially thawed or it will be too liquid to handle. Freshly killed mice, of course, pose no problem in this respect. The brain is transferred to a labeled, sterile, corked, flat-bottomed shell vial, 17 x 55 mm (**Figure 79**) ; then a culture is made by immersing the tip of the scissors into a tube of thioglycollate broth and wiping it against the side of the tube. These scissors and forceps are then set aside to be sterilized. The label on the tube is checked against that on the envelope from which the mouse was taken, since confusion of labels is a serious source of error.

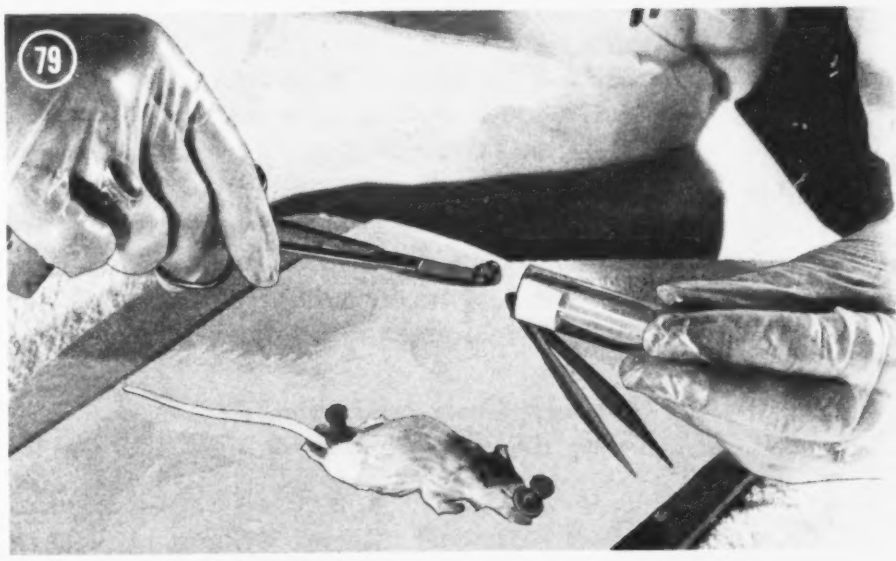
The pins are removed and placed in container of Lysol solution. The mouse carcass is wrapped in the butcher parchment and paper towel and placed in a plastic bag for disposal. The mouse board receives a new set of covering papers, and sterile instruments are used for the next mouse.

The vials containing the mouse brain tissue are accumulated in a refrigerator during the dissection period. These tissues are then either ground immediately into suspensions or are frozen at −70°C and ground at a later date.

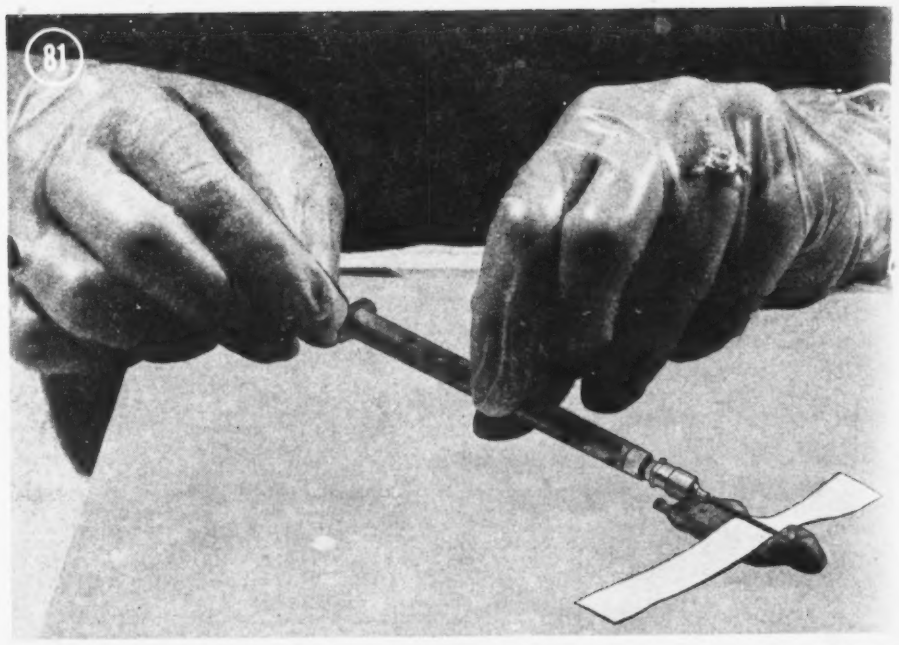
Another method can be used for newborn mouse brain harvesting which is considerably speedier but which requires special caution to preclude dangerous aerosols. Antibiotic-treated 25 percent rabbit serum diluent is distributed to 2 ml amounts into sterile corked Kahn tubes. These tubes are appropriately labeled, placed in a rack, and the rack is set in an ice bath in a safety hood (**Figure 80**).

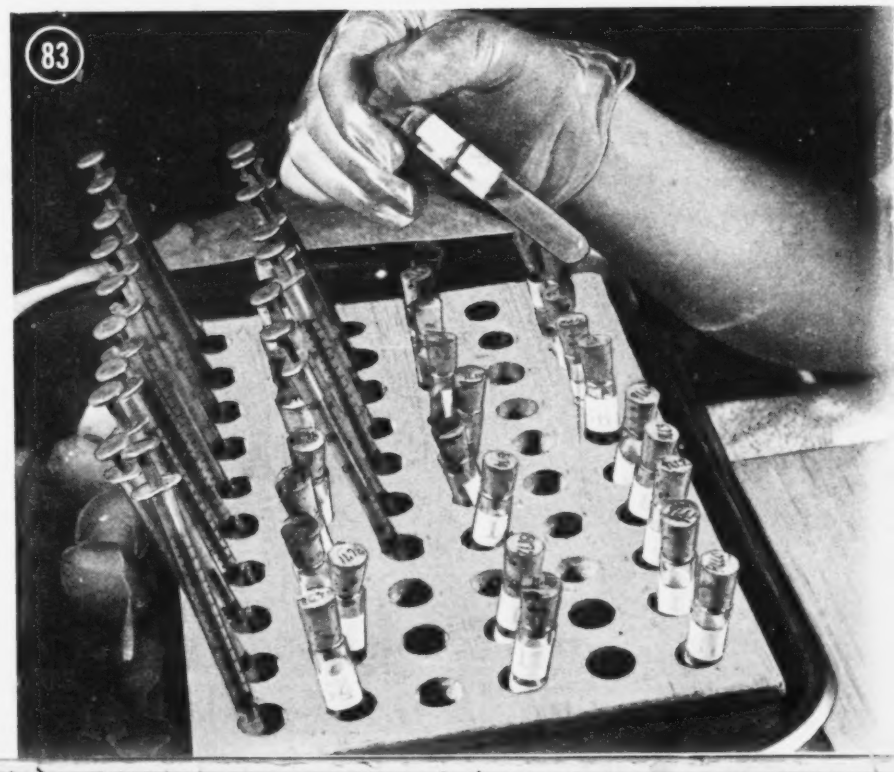
Frozen newborn mice are allowed to thaw completely. With forceps, each mouse to be harvested is placed belly down, head away from the operator, upon a dissecting board covered with a sheet of butcher parchment. A 3″ length of ½″ or 1″ adhesive tape is placed transversely over the mouse to hold it firmly down onto the paper; the leading margin of the tape should be across the shoulders, leaving the head free. The mouse's record number is double-checked against the number of the previously labeled tube. Then





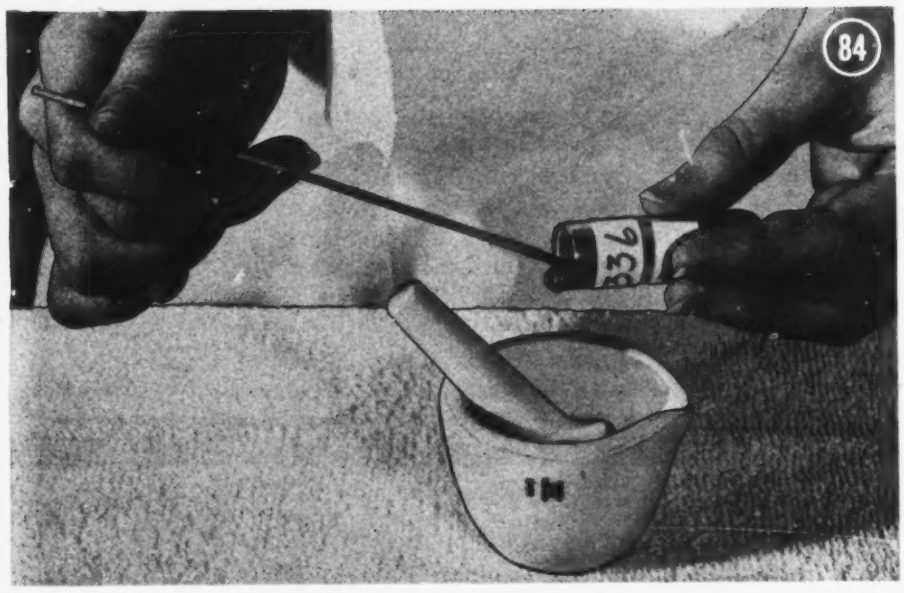
the head and neck are swabbed thoroughly with 1:1000 Merthiolate, and the swab is discarded. Using a disposable 1.0 ml syringe and a 19 or 20 gauge 1″ needle, the cranium is pierced from the back, and 0.1 to 0.2 ml of the viscous brain material is drawn up (**Figure 81**). Disposable syringes are used for greater vacuum. The syringe is inserted into the labeled tube but the contents are not yet discharged. Instead, about 0.5 ml of the antibiotic-treated diluent is slowly drawn into the syringe along with the brain material (**Figure 82**). Then, holding the needle below the level of the remaining diluent, all the material in the syringe is slowly ejected *a single time.* No additional drawing-up and discharging should be done since dangerous aerosols will be produced. The syringe and needle are discarded. *Always use a well-ventilated hood.* Gently shaking the tube after the cork is replaced will assure adequate mixing (**Figure 83**). If virus caused the mouse's death, it will be present in such a high concentration that additional mixing is not required. The suspension is now ready for centrifugation which is described below. If bacterial cultures are desired, insert the needle tip into a tube of thioglycollate medium immediately after the brain harvest and before dilution.






### Grinding, Centrifuging and Passing Mouse Brain Material

Each dissected mouse brain is transferred to a cold, sterile, 3″ mortar with a sterile applicator stick (**Figure 84**) and ground in 2.0 ml (for newborn mouse brain) or 3.0 ml (for weanling mouse brain) of the 25 percent rabbit serum diluent to make an approximate 10 percent brain suspension. Grinding is not required for newborn mouse brains harvested by the syringe technique. Each suspension is poured into a sterile, corked, double-labeled Kahn tube and centrifuged in the cold for 20 minutes at 2500 rpm in an angle-head centrifuge. A separate centrifuge from the one used to spin down the field-collected specimens is employed to minimize the chance of accidental contamination of primary material. The supernates may be poured off into clean corked Kahn tubes and




the labels transferred; however, for routine passages, this step may be skipped. The brain tissue forms such a tight pellet at the bottom of the tube that the clear fluid above may be drawn up into a syringe for inoculation without unduly disturbing it. The suspensions are either inoculated immediately by the intracerebral route into litters of newborn mice or are frozen at —70°C to be inoculated later. If a high proportion of the passages are expected to be virus positive, it is a good practice to intersperse into each inoculation series several normal brain suspensions as controls against careless handling and cross-contamination.

As before, daily checks are made on the mice. In the event of apparent arbovirus isolations, freshly dead, prostrate, or paralyzed mice are saved, and their brains are harvested for virus identification procedures.

**The Use of Cell Cultures for Arbovirus Isolation**

Recently cell cultures have been used more frequently as host systems for the propagation of a number of arboviruses (26-43). A variety of laboratory-adapted arbovirus strains have been studied in avian, mammalian, and arthropod cell cultures. However, the use of cell cultures for routine isolations of arboviruses from field collected materials has not yet been widely accepted. Although no single *in vitro* system has been shown to equal the susceptibility of newborn mice, several cell culture systems have proven useful for specific viruses.

Some of the *in vitro* systems found useful for isolating various North American arboviruses are hamster kidney, monkey kidney, and chicken and duck embryonic cells. The presentation of cell culture methods is beyond the scope of this manual. Therefore, the reader should refer to appropriate references. A partial listing of cell systems found useful for the isolation of certain North American arboviruses of public health importance is presented in the following table.

**Selected Cell Culture Systems Used for Isolation of Certain North American Arboviruses**

| Virus | Duck Embryo | Chicken Embryo | Hamster Kidney | Monkey Kidney |
|---|---|---|---|---|
| EE | 43* | 37,41 | 41,43 | 37,41 |
| WE | 42,43 | 38,39,40 | 38,40,43 | |
| SLE | 42,43 | 38,40 | 38,40,43 | |
| VE | 43 | | 43 | |
| CE | | | 43 | |

*Literature cited.

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# APPENDIX

**1. Supplies for Human Antibody Survey**

Amounts of supplies required are dependent upon sample sizes desired; these are not necessarily the only items or sources which may be used.

a) 20 ml evacuated glass containers (Vacutainer[R] No. 3208, 165 x 16 mm, B-D Company, Columbus, Nebraska).*

b) Vacutainer[R] plastic holders

c) 20 ga., 1½″ Vacutainer[R] needles

d) 10 cc disposable syringe with a 22 gauge needle

e) 2 drams (7.5 ml) screw cap vials

f) Alcohol pledgets

g) Band-aids

h) Adult and pediatric tourniquets

i) Tape board

j) Waterproof adhesive tape, ½″ width

k) Indelible marking pens

l) Disposable scalpels or single-edge razor blades

m) Interview sheets

n) Gum or candy (to reward children)

o) Case for equipment

Before going to the field, several of the evacuated glass tubes in each carton should be checked to make certain they can be filled completely. Some loss of vacuum may occur after storage of three or more years, particularly in the large tubes.

**2. Sizes and Types of Traps useful for the Capture of Various Species of Mammals**

| Type and Size, Inches | Representatives of Species Size | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mice | Rats | Grey Squirrel | Cottontail Rabbit | Skunk or Opossum | Raccoon | Fox | Bobcat | Coyote |
| Sherman | | | | | | | | | |
| 2x2 1/2x6 | X | | | | | | | | |
| 3x3x10 | X | X | | | | | | | |
| 5x5x15 | | X | X | X | | | | | |
| Havahart | | | | | | | | | |
| 3x3x10 | X | X | | | | | | | |
| 5x5x18 | | X | X | | | | | | |
| 7x7x24 | | X | X | X | X | | | | |
| 11x11x36 | | | X | X | X | X | | | |
| 12x12x55 | | | | | X | X | X | | |
| Tomahawk | | | | | | | | | |
| 3x3x10 | X | X | | | | | | | |
| 5x5x16 | | X | X | | | | | | |
| 7x7x20 | | | X | X | X | | | | |
| 10x12x32 | | | | | X | X | X | X | |
| 20x26x60 | | | | | | X | X | X | X |
| Steel Traps | | | | | | | | | |
| #1 | | | X | X | X | | | | |
| #2 | | | | | X | X | X | X | |
| #3 | | | | | | | | X | X |

*The use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

**3. Sources and Approximate Prices of Traps for the Capture of Mammals***

A. Sherman Type

| a) Supplier | Size | Dimensions | Gauge Metal | Price/No. |
|---|---|---|---|---|
| H. B. Sherman | Small | 2x2 1/2x6 ¾ | .020 Aluminum | $220.00/100 |
| P.O. Box 683 | Medium | 3x3x9 | .020 Aluminum | $270.00/100 |
| Deland, Florida 32720 | Large | 5x5x15 | 28 Ga. Galvn. Metal | $550.00/100 |

| b) Supplier | Size | Dimensions | Gauge Metal | Price/No. |
|---|---|---|---|---|
| H. G. Spencer and Sons | Mouse size | 2x2 1/2x6 ¾ | 28 Ga. Galvn. Metal | $2.25/ea. |
| P.O. Box 131 | Rat size | 3x3x10 | 28 Ga. Galvn. Metal | $2.75/ea. |
| Gainesville, Florida 32601 | | | | |

B. Havahart Type

| a) Supplier | Size | Dimensions | Weight | Price/No. |
|---|---|---|---|---|
| Allcock Manufacturing Co. | No. 0 | 3x3x10 | 2 lbs. | $ 3.30/ea. |
| P.O. Box 551 | No. 1 | 5x5x18 | 4 lbs. | $ 5.40/ea. |
| Ossining, New York 10562 | No. 2 | 7x7x24 | 7 lbs. | $ 7.60/ea. |
| | No. 3 | 11x11x36 | 20 lbs. | $15.90/ea. |
| | No. 7. | 12x12x55 | 29 lbs. | $34.20/ea. |

| b) Supplier | Size | Dimensions | Weight | Price/No. |
|---|---|---|---|---|
| Sears Roebuck and Co. | Medium | 7x7x24 | 7 lbs. | $ 6.49/ea. |
| | Large | 11x11x36 | 20 lbs. | $12.69/ea. |

C. National Type

| a) Supplier | Size | Dimensions | Weight | Price/No. |
|---|---|---|---|---|
| | Non-collapsible Traps | | | |
| Tomahawk Live Trap Co. | 101 | 3x3x10 | 2 lbs. | $ 3.45/ea. |
| P.O. Box 323 | 102 | 5x5x16 | 3 lbs. | $ 4.75/ea. |
| Tomahawk, Wisconsin 54487 | 105 | 7x7x20 | 4 lbs. | $ 6.65/ea. |
| | 108 | 10x12x32 | 14 lbs. | $13.25/ea. |
| | 110 | 20x26x60 | 75 lbs. | $45.00/ea. |
| | Collapsible Traps | | | |
| | 201 | 5x5x16 | 3 lbs. | $ 5.75/ea. |
| | 204 | 7x7x20 | 6 lbs. | $ 7.75/ea. |
| | 207 | 10x12x32 | 15 lbs. | $15.95/ea. |
| | 208 | 15x15x42 | 45 lbs. | $27.95/ea. |

*Prices vary with time and supplier.

| b) Supplier | Size | Dimensions | Weight | Price/No. |
|---|---|---|---|---|
| Sullivan's Sure Catch Traps | A0 | 8x8x24 | 3 1/2 lbs. | $ 9.95/ea. |
| P.O. Box 1241 | A1 | 10x10x40 | 13 lbs. | $15.75/ea. |
| 544 South Patterson Street | A2 | 14x14x44 | 17 lbs. | $19.75/ea. |
| Valdosta, Georgia 31601 | A4 | 26x26x72 | 50 lbs. | $49.75/ea. |

D. Steel Traps

| a) Supplier | Size | Price | Type |
|---|---|---|---|
| Woodstream Corp. | No. 1 | $ .52 | Long Arm |
| Front and Locust Streets | No. 2 | $1.31 | Square jaw, coil spring |
| Lititz, Pennsylvania 17543 | No. 3 | $1.66 | Double Arm |
| (Wholesale only, except to government agencies) | | | |

b) Other Sources
Local sports goods or hardware stores

**4. Monel Metal Fingerling Fish Tags**

Supplier: National Band and Tag Co.
Newport, Kentucky 41071
(Various sizes available)

**5. Micro-sampling Pipets**

| Supplier | Size | Catalog No. | Price/No. |
|---|---|---|---|
| Corning Glass Co. | 50 micro liter | 7099-S | $12.39/250 |
| Corning, New York 14830 | 100 micro liter | 7099-S | $12.39/250 |

**6. Portable Dry Ice Chests**

| Supplier | Size | Model No. | Price/No. |
|---|---|---|---|
| Polyfoam Packers Corp. | 5 cu. ft. Royalite Transporter | 50 RS | $ 75.00/ea. |
| 6415 North California Avenue | 5 cu. ft. Royalite Transporter | 50 RR | $127.50/ea. |
| Chicago, Illinois 60629 | 2 cu. ft. Fiberboard Case | 20 FW | $16.25/ea. |

**7. Japanese Mist Nets**

| a) Supplier | Size | Price/No. |
|---|---|---|
| E. A. Bergstrom<br>Northeastern Bird-Banding Association<br>37 Old Brook Road<br>West Hartford, Connecticut 06117 | Type A, (12 meters long ) | $4.10/ea. |

| b) Supplier | Size | Price/No. |
|---|---|---|
| W. B. Davis<br>712 Mary Lake Drive<br>Bryan, Texas 77801 | No. 1, ( 9 meters long ) | $3.00/ea. |

8. **Cannon Netting Equipment**
   a) Cannon Net Supplier: Nichols Net and Twine Co.
   Route 3, Bend Road
   East St. Louis, Illinois 62201
   b) Dill Cannon and Cannon Supplier: Hub City Iron Stove Co.
   Aberdeen, South Dakota 57401
   Price $17.50/ea. plus freight
   c) Black powder charges for cannon nets
   Supplier: Central Technology, Inc.
   Herrin, Illinois 62948
   d) Blasting galvanometer and wire
   Supplier: Obtain locally from hardware store

9. **Bird Bands for Non-regulated Birds**
   Supplier: National Band and Tag Co.
   Newport, Kentucky 41071

10. **Portable generator, Honda E-300, A.C. Single Phase:**
   300 watts @ 117 volts, 60 cycle @ 3,600 RPM;
   D.C.: 100 watts @ 12 volts.
   Supplier: American Honda Motor Co., Inc.
   Price $179.00

11. **Field Diluent**
   Penicillin working stock: To 1,000,000 unit bottle of penicillin G sodium, add 10 ml 0.05 M phosphate buffered saline (pH 7.8) to produce working stock containing 100,000 units per ml.
   Streptomycin working stock: To 5.0 gm bottle of streptomycin sulfate, add 12.5 ml buffered water (pH 7.8) to yield a 400 mg/ml concentrated stock. To prepare working stock containing 40 mg/ml, add 54 ml buffered saline to 6.0 ml of the concentrated stock.
   For 25 percent normal rabbit serum diluent:
   25.0 ml normal rabbit serum
   1.0 ml penicillin (100,000 units/ml)
   4.0 ml streptomycin (40 mg/ml)
   70.0 ml buffered saline (pH 7.8)
   100.0 ml yield